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Conferences and Meetings

1980 July 20–23 International Symposium on "Neuronal Mechanisms of Hearing" to be held in Prague, Czechoslovakia. Information. J Syka, Institute of Experimental Medicine, Czechoslovak Academy of Sciences, 128 08 Prague 2, U nemocnice 2, Czechoslovakia.

1980 September 2–5 IV International Symposium on Facial Nerve Surgery to be held in Los Angeles, California, USA. Further information address Facial Nerve Symposium, c/o Ear Research Institute, 256 South Lake Street, Los Angeles, California 90057 USA.

1980 September 21–22. The XXXI Congress of the Polish Otolaryngological Society will be held in Poznań. Further information Ass Professor Antoni Pruszewicz, Clinic of Otolaryngology Academy of Medicine, 49 Przybyszewskiego Str., 50 355 Poznań, Poland.

1980, September 27–28 The Research Forum, under the joint sponsorship of the Committee for Research in Otolaryngology of the American Academy of Otolaryngology and the Association for Research in Otolaryngology will be held in Anaheim, California. Abstract format instructions from Professor Makoto Igarashi, M.D., Department of Otorhinolaryngology and Communicative Sciences, Baylor College of Medicine, Houston, Texas 77030 USA.

1980 October The Ear Research Institute announces a two-week Temporal Bone Surgical Dissection Course. Information. Antonio De La Cruz, M.D., Director Temporal Bone Surgical Dissection Course, Ear Research Institute, 256 South Lake Street, Los Angeles, CA 90057 USA.

1980 October 3 A Meeting of the O.R.S (Oto-Rhino-Laryngological Research Society) will be held at the Royal National Throat, Nose and Ear Hospital, Gray's Inn Road, London. Information Professor P Stell, Ch.M., F.R.C.S., Department of Otolaryngology Royal Liverpool Hospital, Prescot Street, Liverpool. L7 8XP England.

1981 March 22–27 Second International Conference on Cholesteatoma and Mastoid Surgery to be held in Tel-Aviv Israel. Information Professor J Sadé, 2nd International Conference on Cholesteatoma and Mastoid Surgery P O Box 16271 Tel-Aviv Israel.

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RECOVERY OF THE HUMAN INTRA AURAL MUSCLE REFLEX AFTER "PAUSES" OF VARIOUS DEPTH

E. Borg

From the Department of Audiology and Department of Physiology II Karolinska Institutet Stockholm, Sweden

(Received May 3 1979)

Abstract Decay and recovery of the ipsilateral and contralateral stapedius reflex were studied in eight normal hearing subjects. The activity of the intra-aural muscle was recorded as change of the ear acoustic impedance in response to prolonged stimulation at 2000 Hz. A one-second long attenuation of the stimulus tone by to 40 dB was introduced when the response had decayed to certain level. The recovery following the pause was observed The results show that "pause with depth as small as dB produced approximately 40% of total recovery A 20 dB attenuation resulted in recovery equal to that seen after an interruption of the tone N difference was seen between the ipsilateral and the contralateral reflex The possibility of using stapedius reflex recovery for diagnosis of oto-neurological disorders was discussed.

The stapedius reflex response to a constant sound of more than a few seconds duration adapts gradually This adaptation or "reflex decay is presumably linked to the sensory processes in the afferent auditory pathway and offers a possibility to study certain aspects of processing of acoustic information

Reflex decay may also reveal pathological processes in the region of the auditory nerve (Anderson et al 1969) The more detailed study of the adaptation and recovery processes of the stapedius reflex is also needed in order to understand its possible role in protection from noise-induced auditory fatigue or permanent hearing loss (cf Tonndorf 1976).

It is known that changes of the acoustic stimulus signal counteract the reflex decay (Kato 1913 Wersäll 1958 Gjaevenes & Söhoel 1966 Lutman & Martin 1978 Borg & Ödman, 1979; Borg et al., 1979) The mechanisms for reactivation have been the subject of a series of experiments in which pauses of specified characteristics have been introduced in continuous, decay-producing signals (Borg & Ödman 1979) The aim of the present study was to analyze the role of the depth of the introduced "pause" and in particular to answer the following questions (*a*) How much does a sound pressure need to be lowered in order to result in an observable recovery? (*b*) How great a lowering is needed in order to allow for an optimal recovery?

METHODS

The experiments were performed on eight young adult subjects with no history of ear disease or neurological disease They had normal otoscopic findings and hearing thresholds less than 15 dB (re ISO 1964) between 125 and 8000 Hz. The ipsilateral and contralateral stapedius reflex responses were recorded simultaneously in both ears as changes of the ear's acoustic impedance at 800 Hz (probe tone 70 dB SPL, sound pressure level re 20 μPa, Møller 1961) Pure tones at 000 and 500 Hz (1 s duration and 10 ms rise and fall time) were used as stimuli The sound pressure was individually calibrated by a probe reaching close to the ear drum

The present study has been supported by grants from the Swedish Medical Council (B77-04X-04958-01), the Karolinska Institute and Magnus Bergvall' Foundation

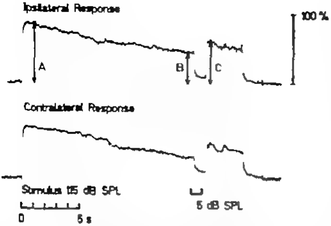

Fig 1 Ipsilateral (above) and contralateral (below) stapedius reflex response to stimulation with 2000 Hz pure tone (115 dB SPL) with a 1 s long 5 dB attenuation of the intensity The maximum response is indicated by the vertical bar (100%) *A*=initial peak amplitude *B*=amplitude immediately before pause" *c*=peak amplitude after pause" Recovery is calculated as $\left[R=\frac{C-B}{A-B}\right]$

Each stimulus lasted 10–20 s and contained only one pause A two-minute interval elapsed between each stimulus In order to minimize transients at the onset and offset of the pause" the stimulus was filtered through a 100 Hz band pass filter (Krohn-Hite model 3202) The ipsilateral and contralateral reflexes were always recorded simultaneously and stimuli were applied alternatingly to the left and the right ear

In addition to the stimulus tone the 800 Hz probe tone was always present in the stimulus ear This tone may influence the reactivation process during the pause In order to investigate this possible interaction one series of measurements was performed with only contralateral reflex recordings and no probe tone in the stimulus ear

In order to establish the stimulus-response function of the reflex 1 second bursts were presented alternately to the left and the right ear The level was raised from 70 dB SPL to about 125 dB SPL in 5 dB steps and thereafter lowered to below the reflex threshold The signal representing the impedance change was recorded on a magnetic tape recorder and later processed During playback the amplitude of the signal was measured at the end of the stimulus and expressed in percentage of maximum obtainable response in the ipsilateral ear Four stimulus response functions were thus obtained two for the ipsilateral and two for the contralateral response at 500 and 2000 Hz each

The adaptation of the reflex (reflex decay) was studied only at 2000 Hz as described by Borg & Ödman (1979) The level of the continuous tone was adjusted (in 5 dB steps) to give an initial impedance change of about 75% of the maximum in the ipsilateral ear When the amplitude had declined to about 40% a 1 s "pause" was introduced The pause consisted of a 1 s decrease of the sound level by 2 dB 5 dB 10 dB 20 dB 30 dB 40 dB or a 1 s interruption of the tone

RESULTS

Typical recordings of ipsilateral and contralateral impedance change signals during activation of the stapedius muscle with a 2000 Hz pure tone with a 1 s "pause" are shown in Fig 1 The pause consisted of a 5 dB decrease in sound level The impedance change reached an initial maximum (*A*) which was followed by a substantial decay During the "pause the impedance changes dropped further and at the reinsertion of the tone the amplitude of the response reached a value (*C*) which was higher than the value immediately before the pause (*B*) This increase of response is evidence for a recovery of the reflex function Recovery was already seen after a 2 dB pause and increased as a function of the depth of the pause for most of the subjects The recovery was quantified by the ratio of the recovered amplitude and the decay $\left[R=\frac{C-B}{A-B}\right]$

The recovery ($R\times100\%$) is shown in Fig. 2A as a function of attenuation of the stimulus in the pause for all subjects (thin lines average is shown by heavy line) It is seen

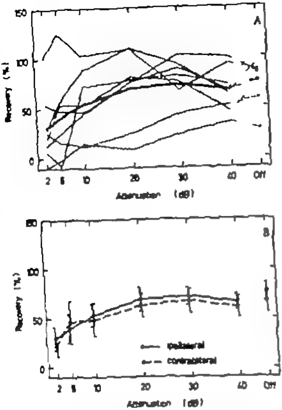

Fig. 2. Recovery (*R* 100%) as function of the depth of 1 long pause at constant 000 Hz pure tone. (A) 8 individual subjects are shown as thin lines, average as heavy line. "Off" shows recovery after turning stimulus off during 1 (equivalent to 60 dB attenuation). (B) Average and standard error of ipsilateral (continuous line) and contralateral (broken line) stapedius reflex response to stimulation with 000 Hz pure tone with 1 long attenuation of stimulus.

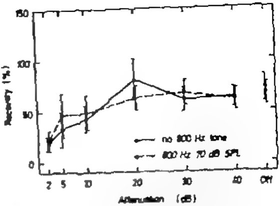

Fig. 3. Recovery of the contralateral stapedius reflex with (broken line) and without (continuous line) 800 Hz probe tone in the *stimulus ear*. *Average* ± S.E.

that recovery increases as a function of depth of the *pause* for all subjects but one.

The individual variability is to some extent due to chance but most likely also to individual factors (cf. Borg & Ödman 1979).

The ipsilateral and contralateral reflexes do not differ systematically (Fig. 2B). For both reflexes a recovery is already seen at 2 dB attenuation (significant <0.05 for the ipsilateral but not for the contralateral reflex, t test). Optimal recovery is on the average present after a 20 dB attenuation.

Although the probe tone was considerably weaker than the stimulus tone (70 dB SPL compared to 100–115 dB SPL) it may influence the recovery during the "pause". One series of measurements was therefore performed only with contralateral reflex recordings and with no probe tone in the stimulus ear. No systematic or significant difference was observed between the results in the two experimental series (Fig. 3).

DISCUSSION

The results show that the decay-recovery mechanism is very sensitive to changes in sound level. A significant recovery is already seen after an attenuation of only 2 dB. Decreasing the sound below a level approximately corresponding to the reflex threshold does not, however, further increase the recovery. *Saturation is reached around 20 dB.*

The functional correlates of the reflex decay and recovery have not been defined at the neuronal level. It is, however, difficult to explain several characteristics of decay and recovery unless it is assumed that they depend on processes *localized* in the afferent part of the reflex, i.e. the cochlea and the lower auditory pathways. The most striking of these properties is the frequency specificity of the decay, limited as it is to high frequencies. A change of stimulus frequency also leads to an immediate recovery of the

response (Gjaevenes & Söhoel 1966) In recordings from auditory nerve fibers Young & Sachs (1973) observed impulse frequency during prolonged constant pure tone stimulation They observed a decay phenomenon similar to a reflex decay and also a recovery after pauses of various duration If one takes into consideration that these experiments were made in anesthetized cats one may conclude that the main part of decay and recovery processes are localized in the inner ear

Young & Sachs (1973) studied recovery of auditory nerve fibers after pauses (tone off) but not after specified reductions in sound level In his work on adaptation in the auditory nerve Smith (1979) does not analyze the influence of attenuation either only of superimposed added signals Neurons in the cochlear nucleus are known however to be very sensitive to amplitude modulation (Møller 1971) of pure tones The sound variations are extremely exaggerated at certain modulation frequencies However direct evidence is lacking that these neurons actually participate in the activation of the middle ear muscles

Observation of stapedius reflex decay has been successfully used in the diagnoses of diseases localized to the auditory nerve and the lower brain stem structures (Anderson et al 1969 and several later studies) It has also been shown in experiments on rabbits that lesions to the auditory nerve and cochlear nucleus give reflex decay and in addition that the recovery after such lesions is very slow (Borg 1976) The diagnostic value of stapedius reflex measurements will probably be further improved by using more elaborate signals such as amplitude modulated sounds One may assume for instance that patients with a pronounced recruitment will be more sensitive to a small amplitude change which could manifest itself as an abnormally large recovery after even a still smaller change of sound level than those showed in the present study

ZUSAMMENFASSUNG

An 8 normalhörenden Versuchspersonen wurde der Verlauf der Abnahme (decay) und Reaktivierung (recovery) des ipsilateralen und kontralateralen Stapediusreflexes durch Aufnahme der relativen akustischen Impedanzänderung bei Stimulation mit einem 2000-Hz-sin-Ton untersucht. In den 2000-Hz Ton wurden dabei Intensitätsverminderungen von 1-sek Dauer und unterschiedlicher Tiefe (im Rahmen von 2 bis 40 dB) eingelegt Es zeigte sich daß bereits nach einer -dB-Verminderung der Stimulusstärke bei der folgenden -dB Steigerung zurück auf die Ausgangsintensität eine gerade merkbare Zunahme der Reflexamplitude festzustellen war Nach einer 1 sek langen 20-dB-Verminderung der Stimulusstärke war die Reaktivierung des Reflexes maximal d h die relative Impedanzänderung war von gleicher Größe wie nach vollständigem Abbruch oder einer 40-dB-Verminderung des 2000-Hz Tones. Zwischen dem ipsilateralen und dem kontralateralen Reflex zeigte sich dabei kein Unterschied. Im Anschluß an diese Befunde wird die Möglichkeit erwogen den Reaktivierungsverlauf des Reflexes in der Diagnostik otoneurologischer Störungen einzusetzen

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Erik Borg, M.D.
Dept of Physiology II
Karolinska Institutet
S 10401 Stockholm
Sweden

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DISPLACEMENT OF THE MALLEUS IN NEONATAL GOLDEN HAMSTERS

Evan M Relkin and James C Saunders

From the Auditory Physiology Laboratory North ·estern University E·anston Illinois and Department of Otorhinolaryngology and Human Communication U iversity of Pennsylvania Philadelphia Pennsylvania USA

(Received August 20 1979)

Abstract A capacitive probe was used to measure displacements of the umbo of the malleus in neonatal golden hamsters for discrete frequencies from 0 6 to 35 0 kHz. Displacement, extrapolated to a sound pressure of 100 dB SPL, plotted as a function of frequency demonstrated low pass characteristics with a cutoff frequency near 9 0 kHz. The amplitude of displacement increased with age to a plateau at all frequencies. Analysis of the data below the cutoff frequency indicated that low frequency displacements were dominated by a compliance which increased with age It was also found that high frequency responses showed evidence of increased mass limitation in younger as compared with older subjects Comparisons between the inverse of velocity and evoked response threshold curves indicated that the middle ear plays an important role in determining thresholds throughout development.

In any experiment in which a sound stimulus is applied to the intact tympanic membrane and middle-ear system the input to the cochlea is the product of the stimulus sound pressure and the transfer function of the middle ear Measurements of function in areas of the auditory system central to the middle ear will always reflect the frequency response of the middle ear Indeed it has been argued that the adult threshold sensitivity curve is largely determined by the shape of the middle-ear transfer function (Dallos 1973 Khanna & Tonndorf 1977 Saunders & Rosowski 1979) In studies of auditory development the situation is further complicated by the possibility that the characteristics of the middle ear might change with age and this problem has been recognized by several authors (Änggård 1965 Brugge et al 1978 Finck et al 1972 Foss & Flottorp 1974 Rose et al 1957 Rubel 1979 Saunders et al 1973 Schmidt & Fernandez, 1963) Nevertheless until recently there have been no investigations that described the development of middle-ear function Relkin et al (1979) reported the development of middle ear admittance in neonatal hamsters for frequencies between 0 8 and 1 8 kHz. Admittance magnitude was found to increase monotonicly with age attaining adult values by approximately 50 days postpartum Conclusions concerning the mechanisms responsible for the admittance changes were limited by the narrow range of frequencies for which admittance could be measured However it was observed that the low frequency admittance was dominated by a compliance which increased with age The increasing compliance was evidence that growth in the volume of the bulla was responsible for the increase in admittance A capacitive probe was developed and used to make broadband measurements of displacements of the umbo of the malleus in neonatal golden hamsters The results are compared to the admittance data and to the

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Portions of these results were presented at the 97th meeting of the Acoustical Society of America (*J Acoust Soc Am* 65 Suppl 1 S9 1979).

The results presented in this paper were included in a thesis by E M R submitted in partial fulfillment of the requirements for the Ph D degree of the department of Bioengineering University of Pennsylvania.

The work described in this paper was conducted in the Auditory Research Laboratory of the Department of Otorhinolaryngology and Human Communication of the University of Pennsylvania.

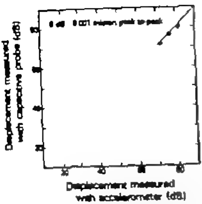

Fig. 1. Correlation between displacements of mini-shaker driven at 1.0 kHz as measured by the capacitive probe and the accelerometer. The diagonal represents perfect correlation.

development of inferior colliculus evoked response thresholds measured in the hamster by Bock & Seifter (1978). The importance of the middle ear in determining developmental threshold sensitivities and possible mechanisms for the changes in middle-ear function are further considered.

METHODS

Apparatus and calibration. A capacitive probe system based on the design of Wilson (1973) was developed and carefully calibrated. For a detailed description of this device and its calibration see Relkin (1979). The frequency response of the capacitive probe system was determined by the beat frequency method (Wilson 1973) and by measuring displacements at the center of the diaphragm of a 12.7 mm condenser microphone (Brüel & Kjaer model 4134) when driven as a speaker. The frequency response of the probe system was flat within ±1.5 dB from 0.030 to 35.0 kHz.

The capacitive probe was mechanically attached to a vibrator which was used for displacement calibration. A variable DC voltage applied to the vibrator allowed fine remote control of the probe-to-umbo distance. Using a microscope equipped with a micrometer eyepiece and stroboscopic illumination, it was established that the capacitive probe output was 100 mV RMS when the vibrator was driven 9.0 microns peak-to-peak at 30 Hz and the resting probe-to-object distance was 55 microns (see Relkin 1979 for details). Thus the probe could be positioned 55 microns from an object by driving the vibrator as described and adjusting the DC voltage to obtain a probe output of 100 mv RMS.

A mini-shaker (Brüel & Kjaer model 4810) equipped with an accelerometer (Brüel & Kjaer model 8000) was further used to measure the probe's dynamic range and linearity. The tip of the capacitive probe was located 55 microns above the endplate of the accelerometer and the shaker was driven at 1.0 kHz. Displacements were measured by both the accelerometer and the probe and Fig. 1 shows a correlation between the two measurements. Measurements made with the capacitive probe were found to be linear and correlated with measurements made with the accelerometer ($r = 0.997$) for displacements between 0.01 and 9.0 microns peak-to-peak giving a dynamic range of 60 dB.

Experiments were conducted within a plexiglass chamber (46 cm W×48 cm H×54 cm D) located within a single-walled sound attenuating room. Acoustic stimuli were produced by speakers mounted on the top of the plexiglass chamber. Several steps were taken to minimize the direct conduction of vibrations to the subject. The plexiglass chamber was situated on two rock slabs placed across a metal desk and was isolated from the rock slabs by foam rubber insulation. The subject was supported by a head holder and platform mounted on an aluminum plate which just fit within the plexiglass chamber. A micromanipulator which held the capacitive probe was also mounted on the aluminum plate. The aluminum plate itself was supported by four acoustic isolation feet (Audio Technica, model AT 605). A control experiment was performed to measure the direct conduction of acoustic vibrations from the speakers to the apparatus. Displacements of a metal cylinder placed in the location to be

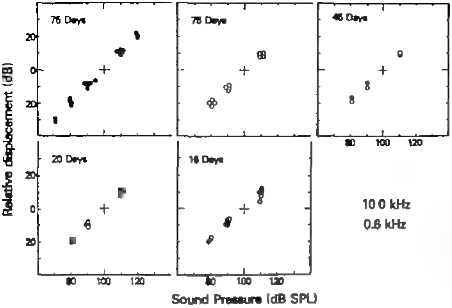

Fig 2 Peak-to-peak displacement of the umbo as a function of sound pressure at 0 6 and 10 0 kHz. Displacements are expressed in dB relative to the displacement at 100 dB SPL for each subject. The data are grouped by age

occupied by the subject s head were measured at approximately the same stimulus sound pressures used to measure umbo displace ments

Procedures Displacement of the umbo was measured in golden hamsters of the LVG LAK strain separated into four age groups 16 20 45 and 75 days postpartum The 75-day-old group was chosen to provide a measure of adult responses Results are re ported for six hamsters at 75 days of age and seven hamsters in each of the other age groups The initial preparation of the subject and the exposure of the bulla have been described elsewhere (Relkin et al 1979) After being anesthetized with an intraperi toneal injection of urethane (1 5 mg/g) the subject was secured in a head holder Body temperature was maintained by a thermostati cally controlled heating pad and an electro-cardiogram was used to monitor the physio-logic condition of the subject The bulla was surgically exposed using a ventromedial ap-proach and nylon tubing (Intramedic Corp PE 10 0 28 mm ID 35 mm L) was tightly inserted into a hole in the bulla to allow equalization of middle-ear pressure to ambient pressure The bony extension of the bulla which covered much of the tympanic mem-brane and defined the terminal zone of the ex ternal meatus (Di Maio & Tonndorf 1978) was removed using a microrongeur Fully ex posing the tympanic membrane facilitated placing the capacitive probe over the umbo The probe tip was visualized under an operat ing microscope and positioned over the umbo using the micromanipulator to which the probe was attached The head holder was then rotated so that the umbo and the tip of the probe were parallel and the probe to-umbo distance was set to 55 microns as described earlier

Pure tones at discrete frequencies between 0 6 and 35 0 kHz were used as stimuli and sound pressure was monitored on-line by a probe tube (5 0 cm L, 0 5 mm diameter) con-nected to a 12 7 mm condenser microphone (Brüel & Kjaer model 4134) The tip of the probe tube was positioned just over the tym-panic ring The sound delivery system was driven at the highest level which did not produce unacceptable distortion or threaten to destroy the speaker system At all frequen-cies no harmonic was less than 40 dB below the level of the fundamental Average sound pressures were between 100 and 120 dB SPL for frequencies less than 30 0 kHz and be tween 90 and 100 dB SPL at 30 0 and 35 0 kHz

Displacement of the umbo and sound pres sure were measured at each frequency for each subject Results were extrapolated to dis

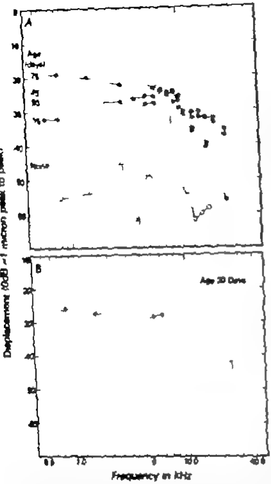

Fig. 3. Peak-to-peak displacement of the umbo at 100 dB SPL as a function of frequency. The parameter is age. Noise level, given by the open circles, represent the total electrical and mechanical noise level of the capacitive probe system. Fig. 3*b* shows standard deviations for the 20-day-old subjects.

placement at 100 dB SPL to provide the frequency response of umbo displacement for constant sound pressure. This extrapolation was justified by examining the linearity of umbo displacement with regard to stimulus level at 0.6 and 10.0 kHz in several hamsters in each age group. The range of the linearity test was limited at the upper bound by the output of the sound delivery system and at the lower bound by the sensitivity of the capacitive probe. Measurements of umbo displacement were made for a range of sound stimuli from zero to −20 to −40 dB relative to the sound pressure used during data collection.

RESULTS

Displacements measured for the metal cylinder represent the total mechanical and electrical noise level of the capacitive-probe system and are shown in Fig. 3 in comparison to measured umbo displacements. Noise levels are not shown for frequencies greater than 17.0 kHz because the output of the capacitive probe did not depend on the level of the stimulus and the extrapolated displacements corresponding to the electrical noise level were outside the range of the figure. It can be seen in Fig. 3 that in the worst case the noise level was 10 dB below the measured displacement and that more typically the noise level was 20 to 40 dB below the measured displacement.

The results of the linearity test are summarized in Fig. 4. Displacement is expressed in dB relative to the displacement at 100 dB SPL for each subject and the data are grouped according to age. Linear regression analysis was applied to the collected data for each age group and test frequency. The correlation coefficient was always found to be greater than 0.98. There was no evidence of nonlinearity at either test frequency for any age group. Therefore the sound pressures used to measure umbo displacement were within the linear range of the hamster middle ear and the extrapolation of measured displacement to displacement at 100 dB SPL was considered valid.

Fig. 3*a* shows the average displacement at 100 dB SPL for each age group as a function of frequency. To give an indication of the variability of the data, Fig. 3*b* further shows the results for the 20-day-old group with error bars corresponding to ±1 standard deviation. The variability at 20 days was typical of that seen in the other groups. The displacement frequency response was a low-pass function

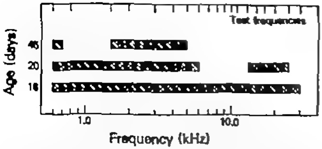

Fig. 4. Summary of the results of *t* tests comparing displacements for each age group to adult displacements. The shaded areas represent ages and frequencies for which displacements were significantly different ($p<0.50$) from the displacement at 75 days at the respective frequency. The test frequencies are indicated at the top of the figure.

with a cutoff frequency near 9.0 kHz for each age group. Displacement increased as a negatively accelerating function of age at all frequencies. A *t* test for independent samples was performed to test whether displacements measured for the 16-, 20- and 45-day-old groups differed significantly from displacements measured for the 75-day-old group. The results of the *t* tests are summarized in Fig. 4. The darkened areas in the figure represent frequency and age combinations for which the average displacement was significantly different ($p<0.05$) from the displacement at the respective frequency in the 75-day old group. The results of the *t* tests confirm the pattern of development that is evident in Fig. 3. At 16 days of age, the displacement at all test frequencies was significantly less than that seen in the 75-day-old hamsters. By 20 days, displacements near the cutoff frequency (7.0–11.0 kHz) were the same as in the adults. However, at lower and higher frequencies the response was still significantly less than in the adult. At 45 days, only frequencies below 5.0 kHz exhibited displacements significantly less than those observed in adults.

Fig. 5 shows the calculated velocity of the umbo as a function of frequency for each age group. The velocity frequency response curves had bandpass characteristics with maximum velocity at a center frequency that corresponded to the cutoff frequency of the displacement curves. Linear regression analysis was used to calculate the slope (in dB/octave) of the low and high frequency skirts of the velocity curve for each age group and the results of these calculations are given in Table I. The low frequency slope did not vary greatly with age and had a value of 5.5 dB/octave (standard deviation = 0.8 dB) averaged over the four age groups. The high frequency slope, however, increased rapidly from −13.6 dB/octave at 16 days to −1.8 dB/octave by 45 days. It can be seen in Table I that the correlation coefficients for the analysis of the high frequency slopes for the two oldest groups were considerably lower than the correlation coefficients for the two youngest groups. The high frequency slopes for the two older groups were almost horizontal and thus the correlation coefficients for these groups were less meaningful. In order to get a better indication of the high frequency slopes, linear regression analysis was further used to calculate the high frequency slope for the displacement data and the results are also given in Table I. The slopes for the displacement data were 6 dB/octave less than the slopes for the velocity data as would be expected.

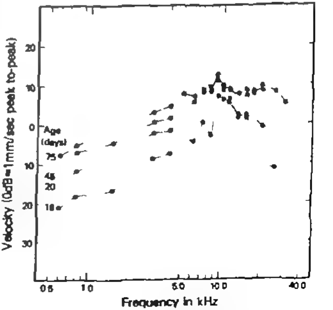

Fig. 5. Velocity of the umbo at 100 dB SPL as a function of frequency. The parameter is age.

Table I *Slopes calculated for velocity and displacement data*

Age (Days)	Slope (dB/octave)	S.E.	
	Low frequency slope calculated for velocity data		
16	5.9	0.7	0.93
20	6.2	0.5	0.98
45	5.2	0.3	0.98
75	4.7	0.3	0.98
	High frequency slope calculated for velocity data		
16	13.6	1.7	−0.96
20	8.7	0.9	−1.97
45	1.8	1.0	−0.67
75	1.8	0.9	−0.56
	High frequency slope calculated for displacement data		
16	19.6	1.7	−0.98
20	14.7	0.9	−0.99
45	7.8	1.0	−0.96
75	7.7	1.1	−0.95

More importantly the correlation coefficients for the analysis of the high frequency displacement slopes were all greater than 0.95

DISCUSSION

Displacement measures of middle-ear function are most commonly made at the stapes since stapes motion describes the input to the cochlea. Stapes displacement differs from umbo displacement as determined by the lever ratio. It has been shown in the guinea pig, a rodent similar to although larger than the hamster, that the lever ratio is largely frequency independent up to 25.0 kHz (Manley & Johnstone 1974). Uncertainty concerning the lever ratio, particularly in younger hamsters, is a complicating factor when considering the present results in comparison to more central auditory responses. There were two reasons for measuring displacement at the malleus in the current study. First, it would have been very difficult, if not impossible, to position the capacitive probe near the stapes in the hamster middle ear. Secondly, and more important, exposing the stapes would have required opening the bulla. Since changes in the middle-ear cavity were thought to play an important role in the development of middle-ear function, it was important not to alter the acoustics of this cavity.

Displacements of the umbo measured in the adult hamster can be compared to displacements measured in the guinea pig (Manley & Johnstone 1974). Displacement of the umbo in the guinea pig at 100 dB SPL with the bulla closed was approximately 0.2 microns peak-to-peak below 3.0 kHz. Above 3.0 kHz, displacement decreased at a rate of 6 dB/octave and above 30.0 kHz, at a rate of 20.0 dB/octave. Displacement of the umbo in adult hamsters was approximately 0.1 micron peak-to-peak at 100 dB SPL up to 9.0 kHz. Above 9.0 kHz displacements in the hamster decreased at a rate of 7.7 dB/octave. Although the low frequency amplitudes were similar for the two species, the hamster had a higher cutoff frequency. The hamster bulla (0.03 cc Relkin 1979) is almost an order of magnitude smaller than the guinea pig bulla (0.25 cc Mundie 1963) and therefore the middle-ear space of the hamster is less compliant than that of the guinea pig. Furthermore, as a consequence of middle-ear size, the ossicles of the hamster are probably smaller and therefore less massive than the ossicles of the guinea pig. Assuming a second order system, the cutoff frequency is inversely proportional to the square root of the product of mass and compliance (Kinsler & Frey 196.., Møller 1972). Thus, decreased mass and compliance might account for the higher cutoff frequency observed in the hamster. Careful examination of the high frequency slope for the hamster suggests that the high frequency rolloff increased above 25.0 kHz. Unfortunately, there were not enough data points above this frequency to do a rigorous analysis of the slope. Measurements could not be made above 35.0 kHz because the sound system could not generate the sound pressures needed to produce measurable displacements.

The low frequency slope of the displace-

ment curve for the neonatal hamsters showed little change with age There was however an increase in the amplitude of the low frequency displacement The average low frequency slope of the velocity curves was 5 5 dB/octave for all age groups A low frequency slope of 6 dB/octave would be expected for a system which is dominated by compliance at low frequencies (Møller 1965 Mundie 1963) The present data indicate that low frequency displacement of the hamster malleus is controlled by a compliance that increased with age The most obvious explanation for an increase in compliance would be an increase in middle-ear volume due to growth of the bulla or reabsorption of embryonic fluids in the middle ear Stephens (1972) has reported residual embryonic fluids in the hamster middle ear up to 20 days of age and this finding was confirmed during the present study The compliance of the tympanic membrane also contributes to the input admittance of the middle ear (Møller 1965 Zwislocki 1962 1963) It is possible that the mechanical properties of the tympanic membrane also change with age A developmental study of tympanic membrane fibers would be useful in testing this possibility

The high frequency slope of −7 7 *dB/octave* in the 45- and 75-day-old animals indicates that the adult middle-ear mechanism may be described by a first order system over the range of frequencies studied In younger animals the high frequency slope was −14 7 dB/octave at 20 days and −19 6 dB/octave at 16 days *These data indicate that the displace*ment response changed from a third order system (i e −18 dB/octave) at 16 days to a second order system (i e −12 dB/octave) at 20 days and finally to a first order system by 45 days The higher order responses in 16- and 20-day-old hamsters were evidence for the existence of additional mass elements in the middle-ear system of these younger animals as compared with the adults (Johnstone & Sellick 1972 Møller 1963)

Stephens (1972) and Arsdel & Hillemann (1951) have reported that ossification of the ossicles in the hamster is not complete until at least 20 days Therefore the composition and hence the mass of the ossicles must be changing at least up to that age It is possible that the immature ossicles simply weigh more perhaps because of residual embryonic fluids or a thicker mucosal lining The effective mass of the ossicular chain could also be increased by a shift of the axis of rotation away from the center of mass or vice versa Such a shift would result in an increased moment of inertia It is possible that the mass of the immature ossicular chain is distributed differently than in the adult Alternatively it might be that the ligaments of the immature middle ear do not maintain the axis of rotation at the center of mass Non-mammalian vertebrates do not possess the elaborate suspension system found in mammals It is interesting to note that the high frequency rolloff of displacement responses in non-mammalian vertebrates was 20 dB/octave (Saunders & Johnstone 1972) This slope is comparable to that measured in the 16-day-old hamsters A histological study of developmental changes in the ossicles and ligaments of the hamster middle ear would be of great help in explaining the changes with *age observed for the high frequency displace*ments

Admittance is given by volume velocity divided by sound pressure and can be compared to velocity of the malleus for low frequencies (Møller 1963 1965) Both admittance magnitude (Relkin et al 1979) and umbo velocity were found to increase with frequency at a rate comparable to 6 dB/octave For the admittance magnitude data the average slope for hamsters 25 days of age and younger (4 6 dB/octave) was found to be significantly less than the average slope for older hamsters (6 3 dB/octave) This difference in slopes was not reproduced by the velocity data The disagreement between the admittance and velocity measures could indicate that portions of the tympanic membrane were not completely coupled to the malleus in

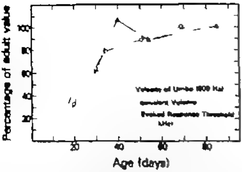

Fig. 6. Comparison between relative growth curves of velocity at 800 Hz, equivalent volume calculated from admittance data (Relkin 1979), and the inverse of evoked response threshold (Bock & Seifter 1978). All values are expressed in percent of the value for the oldest subject in the respective study.

younger hamsters. A similar decoupling was found in adult cats for frequencies above 2.0 kHz (Khanna & Tonndorf 1972; Møller 1963). Nevertheless, both the admittance and velocity measures indicated a compliance which increased with age. Since compliance at the tympanic membrane is thought to be largely due to the middle-ear volume (Mundie 1963; Zwislocki 1963, 196.), it is convenient to express compliance in terms of equivalent volume. The growth curve for the equivalent volume calculated for the admittance data (Relkin 1979) can be compared to the growth curve for the low frequency velocity of the umbo. Fig. 6 shows relative growth curves for the equivalent volume and the velocity measured at 800 Hz. A growth curve for the inverse of the evoked response threshold (Bock & Seifter 1978) is also included in the figure. All values are expressed in percentage of the value for the oldest subjects in the respective study. The similarity among the growth curves is quite evident and suggests a common mechanism.

The inverse of velocity can be compared to threshold sensitivity since velocity of the stapes determines the input stimulus to the cochlea (Dallos 1973; Møller 1963). We recognize that there is uncertainty concerning the transmission of vibrations from the malleus to the stapes in the present study. Never-

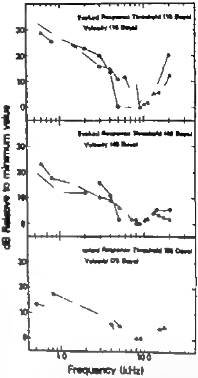

Fig. 7. Comparison of inverse velocity and evoked response thresholds (Bock & Seifter 1978). The data are arbitrarily plotted relative to the respective minimum value.

theless, a comparison between the inverse of umbo velocity and evoked response thresholds measured in the inferior colliculus of the hamster (Bock & Seifter 1978) is instructive. Fig. 7 shows such a comparison for adults and two other groups matched as closely as possible in age. Inverse velocities and thresholds are arbitrarily plotted relative to the respective minimum values. It can be seen that the slopes of the inverse velocity curves and threshold curves are similar in each comparison. Most striking is the agreement of the high frequency slope for the 15/16-day-old hamsters. This slope is obviously greater than the high frequency slope for the older hamsters. The comparisons in Fig. 7 strongly suggest that the middle ear is important in determining the frequency dependency of thresholds in the auditory system throughout development. Therefore, developmental

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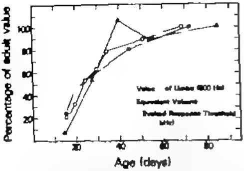
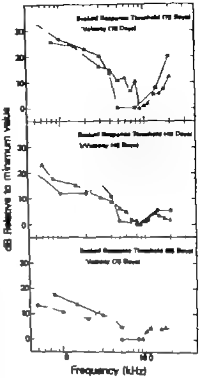


Fig 6 Comparison between relative growth curves of velocity at 800 Hz, equivalent volume calculated from admittance data (Relkin, 1979), and the inverse of evoked response threshold (Bock & Seifter 1978). All values are expressed in per cent of the value for the oldest subjects in the respective study



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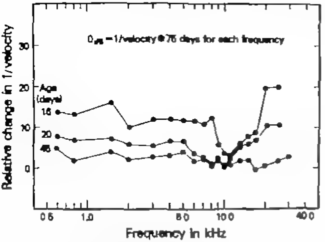

Fig 8 Difference in dB of the inverse of velocity relative to the value at 75 days The parameter is age

thresholds measured within the auditory system with sound stimuli applied to an intact middle ear are determined at least in part by middle-ear development as well as by development of the structure being studied

Fig 8 shows the inverse of umbo velocity for each age group relative to the value at the respective frequency for the adult The results presented in Fig 8 and Table I show that development proceeded most rapidly near the most sensitive frequency (9 0 kHz) and most slowly at the lowest frequencies It has been demonstrated in many species that while the cochlea develops morphologically from base to apex threshold sensitivities generally develop from low to high frequencies (see Rubel 1978 for a review of the pertinent literature) One explanation offered for this discrepancy is that the immature middle ear selectively attenuates high frequencies (Brugge et al 1978 Rubel 1978 Saunders et al 1973) Displacements measured at the umbo of the hamster do not support this hypothesis Perhaps the answer to the problem lies in the characteristics of the ossicular chain or in the cochlea itself

It must be said however that the middle ear cannot as yet be discounted when considering the problem of low to high frequency development of threshold sensitivities Although the velocity data do not show a low to high frequency pattern of development neither do the evoked response thresholds of Bock & Seifter (1978) The present study and the Bock & Seifter (1978) study began at 16 and 15 days of age respectively Up to 15 days the hamster bulla is completely filled with a thick mesenchymal fluid (Stephens 1972) Umbo displacements in subjects less than 16 days of age were smaller than the resolution of the capacitive probe system The authors would like to suggest that the low frequency high threshold sensitivity curves reported for the youngest subjects of various species might have been measured before the middle ear was free of mesenchyme It is true that the penetration of sound into a fluid decreases with frequency After some early age most developmental threshold curves do show a pattern which is similar to the present inverse velocity data and the evoked response data of Bock & Seifter (1978)

ZUSAMMENFASSUNG

Eine kapazitive Sonde wurde im Versuche verwendet, um die entstehenden Verschiebungen im Umbo des Malleus in neugeborenen Goldhamstern zu Einzelfrequenzen von 0 6 bis 35,0 kHz zu messen Die zu einem Schalldruck von 100 dB SPL extrapolierte Verschiebung zeigte als Funktion der Frequenzlänge eine Tiefpaßeffekte mit einer Grenzfrequenz um 9,0 kHz. Das Verschiebungsausmaß nahm mit dem Alter des Versuchstieres zu bis es zu allen Frequenzen ein konstantes Niveau erreichte Eine Analyse der unter der Grenzfrequenz liegenden Daten wies darauf hin daß Verschiebungen zu niedereren Frequenzen von einer mit dem Alter zunehmenden Federung beherrscht wurden Dabei wurde festgestellt daß, im Gegensatz zu älteren die Reaktionen jüngerer Versuchstiere auf höhere Frequenzen eine zugenommene Masse zeigten Vergleiche zwischen der Umkehrung der Geschwindigkeit des Malleus und der Schwellenkurve der hervorgerufenen Reaktionen wiesen darauf hin daß das Mittelohr im Laufe des physiologischen Entwicklungsprozesses eine bedeutende Rolle bei der Determinierung der Hörschwellen spielt

ACKNOWLEDGEMENTS

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Dr Evan Relkin Ph D
Northwestern University
Auditory Research Laboratory
Frances Searle Building
2299 Sheridan Road
Evanston Illinois 60201
USA

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TYMPANIC MEMBRANE CHANGES AFTER INSTALLATION OF POLYETHYLENE GROMMETS

An Experimental Study in the Rat

L E Stenfors and B Winblad

From the Departments of Otorhinolaryngology and Pathology University Hospital of Umeå Umeå Sweden

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Abstract The object of this study was to establish the morphological changes which occur when a polyethylene grommet is implanted in the normal tympanic membrane of experimental animals We found a serous effusion around the grommet and in the attic during the first weeks after installation All the grommets had been displaced away from the tympanic memebrane in a medial direction into the middle ear cavity The displacement was most likely brought about by a squamous cell epithelial and connective tissue hyperplasia which reached its maximum after –3 weeks Neither atrophy atelectasis nor retraction was observed whether in the pars flaccida or the pars tensa A greyish horseshoe-shaped configuration was seen in all specimens in the undisturbed front quadrants No firm histo-pathological correlation was found to explain these changes. In these studies in the rat, the perforation appeared to heal although delayed in the same way as after a central perforation once the polyethylene grommet had been ejected

The surgical treatment of secretory otitis media dates back to the end of the 19th century when Adam Politzer presented myringotomy and air insufflation in the middle ear as a method of treatment Armstrong (1954) introduced a new form of treatment for chronic secretory otitis media in which he installed a vinyl tube (inner diameter 1 5 mm) in the tympanic membrane The intention was to maintain continuous ventilation of the middle ear and to permit drainage of fluid into the external auditory canal Armstrong recommended that the tube should be held in place in the tympanic membrane for 2–3 weeks but warned that a permanent perforation of the tympanic membrane could arise if the implantation is prolonged

A number of studies have been published recording treatment results with plastic tube insertion in the tympanic membrane in cases of chronic secretory otitis media in man (Mac Kinnon 1971 Kilby et al 1972 Mawson & Fagan 1972 Hussl 1973 Kokko 1974 Sadé & Berco 1976 Gundersen & Tonning 1976 Birck & Mravec 1976 Blatnik et al 1977 Adkins 1977)

As far as we know no experimental studies on animals have yet been published regarding the morphological changes which arise in the tympanic membrane after insertion of these plastic tubes Consequently it is not known whether tympanic membrane changes are caused by the chronic middle ear disease itself or by the foreign body in the membrane The aim of the present study was to determine the effects of a polyethylene grommet implanted in the structure of the normal tympanic membrane in the rat

MATERIAL AND METHODS

Rats (Sprague Dawley) weight approx 400 g were used as experimental animals The animals were kept under standard laboratory conditions They were anesthetized by intraperitoneal injection of Ketalar® 150 mg/kg body weight The circumference of the installed polyethylene fragment was 1/4 of the circumference of the polyethylene tubing used in all ventilating tube installations performed

Table I *Macroscopic and microscopic findings in rat tympanic membranes after installation of polyethylene grommets*

Time for autopsy	1 h	3 d	1 w	2 w	3 w	5 w	7 w	10 w
No. of tympanic mem- LM	3	3	2	7	5	5	2	2
branes investigated SEM	3	2	2	2	3	–		1
Macroscopic findings								
Number of ejected/inserted grommets	0/42	0/36	0/31	0/27	4/18	3/10	3/5	3/3
Perforation (open – blocked)	open	open	open	blocked	blocked	blocked	blocked	blocked
Hyperemia (– + ++)	–	+	++	++	+	–	–	–
Effusion in attic (++)	–	+	++	++	–	–	–	–
Horseshoe-shaped structure in pars tensa (+)	–	–	–	+	+	+	+	+
Light microscopic findings								
Inflammatory reaction (+ +)	–	++	++	++	+	–		–
Squamous cell hyperplasia (– ++)	–	+	++	++	++	++/	++/	–
Connective tissue hyperplasia (+ ++)	–	–	+	++	++	+	+	+

Hyperemia in the pars flaccida and on the handle of the malleus

here the grommets were still in place—healed tympanic membranes.

at the ENT Department, Umeå University Hospital. Thus the installed plastic material lacked a lumen but did have an outer and inner flange. Preliminary trials with small tubes suitable for the tympanic membrane of the rat showed that they all became occluded within a day. In 20 rats polyethylene grommets were installed in the right tympanic membrane. On the left side only myringotomy was performed. In 11 rats polyethylene grommets were installed in both ears. The grommet was installed in the rear upper quadrant. The rats were anesthetized twice weekly and the membranes inspected. After various intervals (see Table I) some animals were decapitated and the tympanic membranes with surrounding annular ring excised and fixed in ice-cold Karnovsky's solution (paraformaldehyde–glutaraldehyde) for 4 hours. The specimens (29) for light microscopy (LM) were decalcified for 24 hours in New Decalc® (manufactured by Histolab, Betlehem Trading, Göteborg, Sweden) and embedded in paraffin wax after the polyethylene grommet had been removed from the membrane. The sections were stained with hematoxylin-eosin and according to van Gieson.

The specimens (13) for scanning electron microscopy (SEM) were dehydrated in increasing concentration of ethanol and ethanol–amylacetate up to a concentration of 100% amylacetate and dried with the 'critical point' drying procedure. The specimens were then mounted on stubs and under continuous rotation and tilting covered with an approx. 200 Å thick layer of gold. The specimens were inspected using a Cambridge Stereoscan S4 scanning electron microscope.

RESULTS

Macroscopic and scanning electron microscopic findings (SEM)

Upon inspection ½ an hour after implanting of the polyethylene grommet the perforation could not be visualized via the auditory meatus as the outer flange of the grommet covered the edges. When the tympanic membrane was viewed from the middle ear side, however, after being removed from the temporal bone

Fig 1 Scanning electron microscopic (SEM) picture of a right rat tympanic membrane viewed from the medial side ½ an hour after installation of a polyethylene grommet The medial flange of the grommet (*pg*) quarter-size of an ordinary ventilating tube is seen in the upper rear quadrant. Pars tensa (*pt*) Handle of malleus (*mm*) ×20

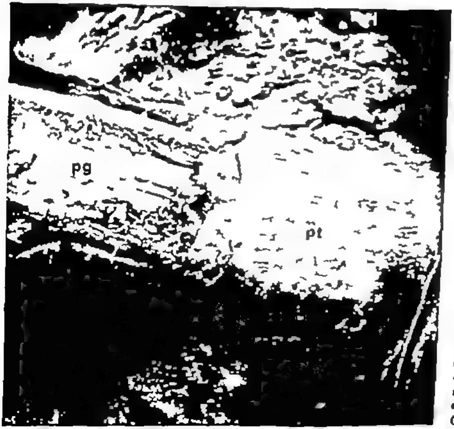

Fig 2 SEM weeks after installation The tympanic membrane viewed from the medial side The perforation is healing along the grommet and the opening is completely closed by the polyethylene piece and the healing membrane Polyethylene grommet (*pg*) Pars tensa (*pt*). ×50

Fig. 3. SEM 3 weeks after installation. I the tympanic membrane the outer flange of the grommet has partly passed through the perforation and is being pushed into the middle ear cavity 20.

it could be seen both macroscopically and in SEM that the perforation was clearly larger than the grommet (Fig. 1)

After 3 days the vessels on the handle of the malleus and in the pars flaccida were distended and there was some thin yellow effusion around the plastic piece and in the attic However this effusion disappeared after 3 weeks when the perforation was blocked

The perforation remained open that is there was a split between the grommet and the perforation edge for 1 week After 2 weeks it was observed macroscopically and by SEM that the healing membrane was growing closely along the polyethylene grommet in a medial direction and there was no passage of air through the tympanic membrane (Fig. 2)

After 17 days the first grommet was ejected from the tympanic membrane and pushed into the middle ear cavity In this animal the grommet in the other tympanic membrane was then in the process of being ejected from the membrane (Fig 3) In the remaining animals the grommets were ejected between the 3rd and 7th week and after 10 weeks all grommets lay inside the middle ear after which delayed perforation healing occurred

After 2 weeks and up to 10 weeks there could be observed in all membranes in the untouched front quadrants a greyish, opaque horseshoe-shaped structure which lay parallel with the annular ring anterior malleolar fold and down parallel in front of the handle of the malleus

Light microscopic findings (LM)

In LM an inflammatory reaction could be observed after 3 days in the edges of the perforation with an accumulation of inflammatory cells mainly granulocytes and fibrin (Fig. 4) This inflammatory reaction disappeared gradually and after 3 weeks very few inflammatory cells could be observed The squamous epithelium of the perforation edge was somewhat hyperplastic after 3 days (Fig. 5) and this hyperplasia remained as long as the grommet

Fig 4 Light microscopic (LM) picture 3 days after installation of a *polyethylene grommet The grommet* was removed before preparation for LM The squamous epithelium is retracted and thickened near the perforation and the collagen is crenelated Granulocytes are observed at the edges of the perforation (*per*) External auditory canal (*ea*) Middle ear (*me*) Htx-eosin ×90

was in place in the membrane After 10 weeks, when all grommets had been ejected and all perforations were healed no squamous cell hyperplasia could be seen at all The collagenous elements in the lamina propria of the membrane were initially crenelated and crooked into the middle ear After 1 week there was noticeable hyperplasia (Fig 6) in the connective tissue reaching its maximum after 2–3 weeks (Fig 7) After 10 weeks the scar in the tympanic membrane was still thickened due to the increased inlay of connective tissue in the lamina propria (Fig 8)

Control tympanic membranes with only myringotomy in the upper rear quadrant were all healed after 7–10 days Initially the perforation was bridged over by fibrinous material followed by squamous cell epithelium on the stromal connective tissue

DISCUSSION

It seems unlikely that a foreign body in this case a polyethylene grommet would not in the *long run leave some trace of a disturbance on* the structure of the tympanic membrane In

Fig 5 LM 7 days after installation The edge of the perforation is bent towards the middle ear and covered by hyperplastic squamous epithelium. Htx-eosin ×60

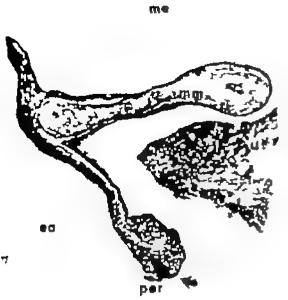

Fig. 6. LM 2 weeks after installation. The edge of the perforation is thickened and covered by hyperplastic squamous epithelium. In the subepithelial tissue some inflammatory cells are observed. The mucocutaneous junction is easily recognized (*arrow*). Perforation (*per*). Middle ear (*me*). External auditory canal (*ea*). van Gieson, ×125.

our experiments an irritative reaction was seen—at least initially—in the mucous membrane of the middle ear, with dilated vessels in the pars flaccida and a thin yellow serous effusion in the attic and around the polyethylene grommet. Birck & Mravec (1976) stated that ear discharge was the most usual complication with plastic tube installation and that this phenomenon occurred on 15.1% of cases. In our investigation the effusion disappeared after 2–3 weeks, at which time the perforation was closed, that is, there was no split between the grommet and the perforation edges and no possibility of air passing through the tympanic membrane.

According to MacKinnon (1971) horseshoe shaped hyaline-like structures were seen in 33% of cases with plastic tube installation. These were most likely caused by the chronic secretory otitis and could be reversible. Mawson & Fagan (1972) described changes similar to those seen in tympanosclerosis in 60 tympanic membranes out of 202 with secretory middle ear otitis treated with ventilating tubes. In the present study we saw in all rats a grey opaque horseshoe-shaped structure in the front quadrants of the membranes. These alterations arose after 2 weeks and were still there after 10 weeks. They were also seen in isolated cases in the control tympanic membranes which had only been subjected to myringotomy.

Histological examination did not reveal any hyaline structures, a finding in agreement with the macroscopical results. In some places, however, squamous cell epithelial hyperplasia was observed in front of the handle of the malleus and parallel with the annular ring. The macroscopical findings often disappeared upon dissection and preparation of the membrane. Our investigations indicate that the greyish deposits in the membrane are not necessarily *sequelae post otitidem* (Ferlito 1979) but could be a consequence of, for example, tension changes in the membrane.

We could not find any atrophic scars in the membranes. Kilby (1972) reported that atrophy was three times more common in membranes treated with a plastic tube than after frequent myringotomy. MacKinnon (1971) reported that the membrane became thinner and more atrophied, with a reduced lamina propria, after plastic tube treatment. By contrast, we observed in rats that the membrane was clearly thickened, especially in the lamina propria, after 10 weeks, a finding corresponding to a normal traumatic tympanic membrane perforation which healed after 10 days.

Kilby et al. (1972) indicated that retraction of the attic and the upper rear quadrant was more common in plastic tube treated membranes than in cases where chronic secretory otitis was treated by myringotomy alone. Mawson & Fagan (1972) could in addition demonstrate retractions in the pars tensa and attic after plastic tube installation. In our investigation in rats no changes were observed

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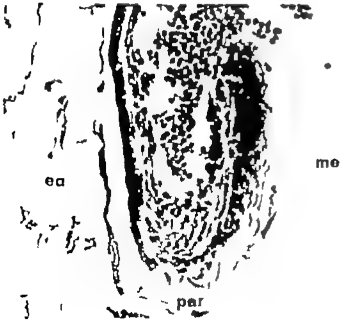

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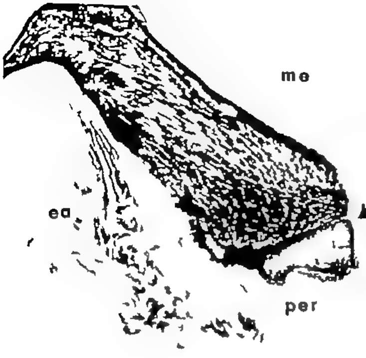

Fig 7 LM 3 weeks after installation. The tympanic membrane close to the perforation is marked by thickened squamous epithelium and stroma hyperplasia. A sparse inflammatory reaction is seen Mucocutaneous junction (*mcj*) m Gieson ×150

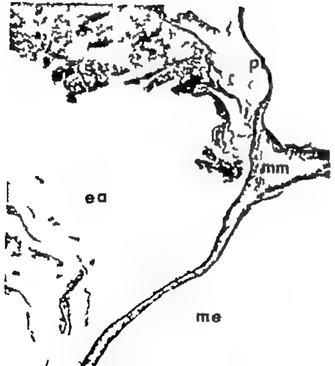

Fig 8 LM 10 weeks after installation Pars tensa (*pt*) is of normal thickness above the handle of the malleus (*mm*) but thickened beneath the handle The lamina propria is thickened There is no squamous epithelium hyperplasia Htx-eosin ×90

in the pars flaccida—apart from distended blood vessels and even this sign disappeared when the effusion in the attic disappeared in conjunction with blocking of the perforation Thus we found no retraction in the pars flaccida or the pars tensa Neither was there any retraction when the perforation was closed and the normal ventilation mechanism via the Eustachian tube was re-established

No permanent perforation could be detected in our material in rats after the grommet had dislodged 3–7 weeks after insertion In man MacKinnon(1971) Kilby et al (1972) Kokko (1974) and Birck & Mravec (1976) have reported permanent perforation in isolated cases after plastic tube installation Armstrong (1954) also warned about this possibility when he described the treatment principle Control membranes in which only perforation was made healed after 7–10 days leaving a greyish opaque scar It seems as if the perforation healed quite normally though later after polyethylene grommet had been displaced away from the membrane On the perforation edge in cases where the grommet still remained in

the membrane after 7 weeks, squamous cell epithelial hyperplasia could be seen. The epithelial cells were still capable of growth and could cover the perforation. Such a squamous cell epithelial hyperplasia was not described by Pulec & Kinney (1973) or Schuknecht (1974) in cases of permanent central eardrum perforation.

We could not find any sign of cholesteatoma formation in our investigation in rats, such as some authors have reported in homo during ventilating tube treatment (MacKinnon 1971, Mawson & Fagan 1972, Kokko 1974, Glasgold 1974).

All the polyethylene grommets had sooner or later fallen out of the tympanic membrane and lay in the middle ear cavity. It could, of course, be thought that the polyethylene grommet followed the epithelial migration laterally as reported by Stinson (1936), Litton (1963, 1968) and Alberti (1964) among others. Hussl (1973) suggested that the epithelial migration was powerful enough to dislodge and eject the ventilating tube. Boedts & Kuijpers (1978) suggested that "the displacement and even extrusion of eargrommets might be at least partly attributed to migration of the upper layer of the stratum corneum". Our investigations, however, showed that the tympanic membrane in rat healed by new tissue formation of the perforation borders and this tissue growth followed the polyethylene grommet in a medial direction and the grommet was pushed by this regenerative process into the middle ear cavity. In homo, however, most tubes are displaced in a lateral direction into the external auditory meatus, although Birck & Mravec (1976) reported in their material fourteen cases where the ventilating tube was found in the middle ear on follow-up. One could then pose the question: "How many of the tubes which are not found at all are in the middle ear cavity?"

ACKNOWLEDGEMENTS

This work was supported by grants from the Medical Faculty, University of Umeå and the Mångberg Fund, Umeå. For technical assistance we are indebted to Bengt Carlöf, Per Hörstedt and Ann-Britt Petersson.

ZUSAMMENFASSUNG

Ziel dieser Untersuchung war festzustellen, welche morphologischen Veränderungen entstehen, wenn ein Polyäthylenröhrchen in gesunde Trommelfelle von Versuchstieren implantiert wird. Man fand eine seröse Effusion rund um das Röhrchen und im Atticus in den ersten Wochen nach der Implantation. Keine Atrophie, Atelektase oder Retraktion wurde beobachtet, weder in der Pars flaccida noch in der Pars tensa. Eine gräuliche hufeisenförmige Konfiguration wurde jeweils in den unverletzten Quadranten gesehen. In diesen Veränderungen konnte kein histopathologisches Korrelat gefunden werden. In diesen Studien an der Ratte wurden die Kunststoffröhrchen in das Mittelohr ausgestoßen, wonach die Perforation erstaunlich zu heilen schien, mit einem ähnlichen Endergebnis wie nach einer zentralen Perforation. Der Abtransport wurde wahrscheinlich von einer kräftigen Plattenepithel- und Bindegewebshyperplasie verursacht, die nach bis 3 Wochen maximal war.

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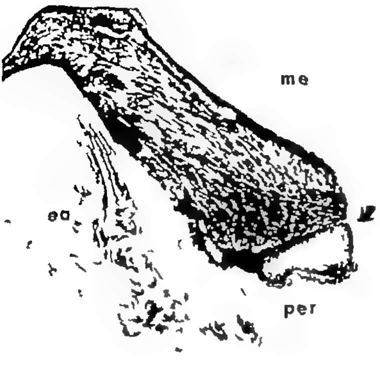

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ELECTRICALLY AND ACOUSTICALLY EVOKED BRAIN STEM RESPONSES IN GUINEA PIG

Kiyofumi Gyo and Naoaki Yanagihara

From the Department of Otolaryngology, Ehime University School of Medicine, Ehime, Japan

(Received June 1[illegible], 1979)

Abstract. Brain stem evoked responses (BSERs) to acoustic and electrical stimulations of the cochlea were compared in guinea pigs. The electric stimuli evoked an averaged response of five to six successive waves within 10 msec, which was similar to that elicited by acoustic stimuli, though the latency of the electrical response was markedly shorter. Moreover, direct electrical stimulation of the auditory nerve evoked the same pattern of response as that elicited by electrical stimulation of the cochlea. On the other hand, stimulation of the vestibular nerve evoked a distinctly different pattern of response that lasted at most for 1.5 msec. Facial nerve stimulation evoked no brain stem response by this procedure. These results suggest that the waves of longer latency over 1.5 msec are available as indicators of auditory activity, even if the response elicited by electrical stimulation of the cochlea might be the sum of auditory and vestibular activities.

Recent investigations in cochlear implant are very exciting, but the final goal of providing a deaf patient with hearing adequate for speech communication has not yet been reached (Tonndorf 1977, Ballantyne 1978). Before clinical use in man, most of the problems should be solved with animal experiments. Though numerous animal experiments have been reported in relation to cochlear implant, all the examiners have experienced difficulty in ascertaining what types of auditory sensation were actually evoked in these animals by electrical stimulation. The indicators of animal responses so far reported are (1) single unit activities from nerves or nuclei on the auditory pathway (Kiang & Moxon 1972, Simmons & Glattke 1972, Schindler et al. 1977), (2) evoked field potentials (Walloch & Cowden, 1974, Simmons 1979), (3) conditioned reflex responses (Clark et al. 1977), etc.

In this work, we studied the brain stem evoked response (BSER) to electrical stimulation of the cochlea. As BSER is thought to be one of the best objective indicators of hearing and is easily recordable with minimum damage to the animal, it would seem worth while to study how BSER can be useful as an indicator of animal response in cochlear implant experiments.

MATERIALS AND METHODS

Twelve normal guinea pigs weighing between 450 and 950 g were anesthetized with Thiamylal sodium (20–30 mg/kg i.p.). During the experiment, the anesthetic was supplemented as required. Lidocaine chloride was also used for local anesthesia. In all animals, tracheotomy was not performed. The head of the animal was secured in a holder. A skin incision was made sagittally along the dorsal of the head to expose the frontal, parietal and occipital bones. A hole 1 mm in diameter was drilled on the vertex 5 mm caudal to the front-parietal suture. Through the hole, a silver-ball electrode was lowered into the dural surface and was fixed to the bone with a binding agent. This vertex electrode served as an active lead. Two other electrodes were implanted into the frontal (ground) and occipital (reference) bones (Fig. 1). All electrodes were placed in the midline to record the BSERs with the

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D Lars-Eric Stenfors
Department of Otorhinolaryngology
University Hospital of Umeå
S 901 87 Umeå
S eden

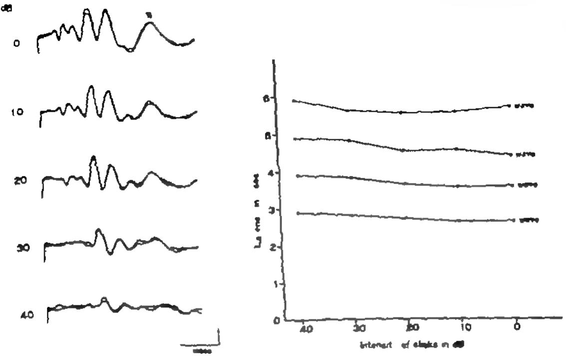


Fig. 3. Acoustic BSERs in normal guinea pig and its intensity-latency relationship. Only waves III–VI were chosen for this study. Latencies of these responses were corrected for the acoustic delay of the stimulating system.

adopted the shorter duration electric pulse. Both acoustic and electric stimuli were given at the same stimulus interval of 80 msec. and their responses were recorded in the same sequence, sweep time, amplification and summation times. Their responses were amplified by a bioamplifier (Dana Japan DA 5?) through a bandpass filter of 300–3 000 Hz and 600 consecutive responses were averaged by a signal processor (SANEI 7SO6). Recordings were repeated twice and the results of the averaged responses were printed out double with an X-Y recorder. The waves of the acoustical responses were presented sequentially with roman numerals, and those of the electrical responses were presented with arabic numerals.

RESULTS

In all recordings, positivity of the vertex electrode relative to the occipital electrode was plotted upwards. Different from a more conventional vertex-mastoid recording of the acoustic response, the vertex-occipital recording showed a phasic wave I and small amplitudes of waves I and II (Fig. 2). This did not obstruct comparison of the acoustical response with the electrical one, for the amplitudes of waves III and IV were large in vertex-occipital recording as well as in vertex-mastoid recording. Moreover, wave VI was prominent in vertex-occipital recording, which was also available for comparison with these responses. Thus waves III, IV, V, VI were chosen for study, and the peak latencies of these waves were measured for each trace. Though wave V was small in amplitude, it served as a good indicator of the response between waves IV and VI.

Fig. 3 shows an example of the acoustical BSER in a normal guinea pig, and illustrates the relationship between the stimulus intensity and response latency. With the same experimental animal, Fig. 4 shows the responses

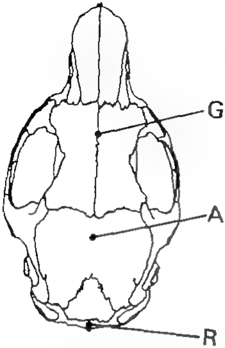


Fig 1 Top view of the skull of a guinea pig and the positions of the recording electrodes Three electrodes were implanted in the midline of the frontal (*G* ground) parietal (*A* active) and occipital bone (*R* reference)

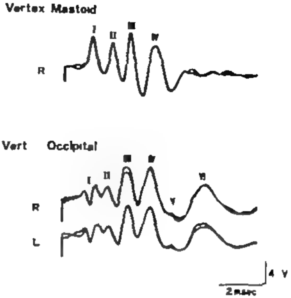


Fig 2 Comparison of acoustic BSERs in different recording positions In conventional vertex-mastoid recording waves I–IV were prominent while waves V and VI were small or unrecognizable In vertex-occipital recording waves III IV and VI were prominent, while waves I and II were small Wave I was presented as a phasic response Wave V was small but was more recognizable in this lead There were no significant differences between the responses evoked by acoustic stimulation of either side Acoustic stimuli were clicks at 100 dB SPL, *R* indicates right ear *L*, left ear

same contribution from either side From these electrodes we could record the acoustical response from one side and the electrical response from other side which seemed valuable for long term observation of the electrical responses in animals As the acoustical response from the unimplanted side served as a good control we could examine the electrical reactivity of the cochlea irrespective of the change in impedance between the recording electrodes which might vary with time

After recording the BSERs to acoustic stimuli the cochlea was exposed through the postauricular approach and the ossicles were carefully removed Using a micromanipulator one stimulating electrode was placed into the scala media through the 0 1 mm diameter hole drilled into the lateral wall of the third turn while the other electrode was placed into the scala tympani through the round window or through a hole drilled in the same way into the wall of the basal turn These stimulating electrodes were made of enamel-coated tungsten wire except for their sharpened tips All the electrical stimulations were performed with these two electrodes

BSER recordings were performed in the following sequence of stimulations (1) acoustic stimulation of each side (2) electric cochlear stimulation by the above mentioned method (3) electrical stimulation of the horizontal portion of the facial nerve (4) electrical auditory nerve stimulation at the modiolus (5) electrical vestibular nerve stimulation at the orifice of the internal auditory meatus

For the acoustic stimulus 4 k Hz clicks with a duration of 0 3 msec were given through a condenser earphone (DCR-48) set up 4 cm away from the tympanic membrane We arbitrarily set the output of the tone generator (Dana Japan DA 502A) at 100 dB SPL as 0 dB On the other hand the electric stimulus (SANEI 3F–36) was a square wave with a duration of 0 01 msec To reduce the interference effect of the electric stimulus we

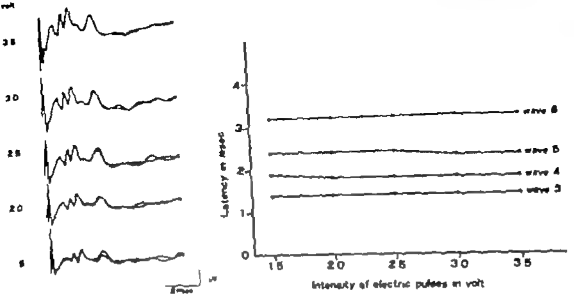


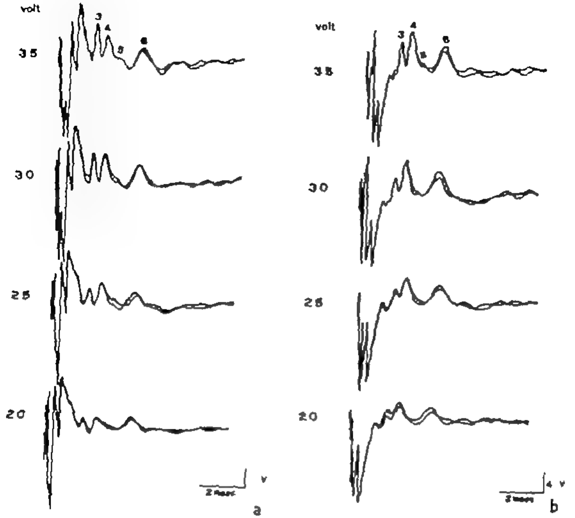
Fig. 5. BSERs by electrical stimulation of the auditory nerve. This procedure evoked a similar pattern to the response elicited by electrical stimulation of the cochlea as shown in Fig. 4.

ulus artifact was so large that the earlier waves might have mingled with it. The origin of the electrical BSER has not been discussed and it is unclear whether all components of the electrical BSER correspond to those of the acoustical response. As waves III and IV of the acoustical response are the most prominent and stable even in reaction to weak stimuli it may be possible to regard waves 3 and 4 of the electrical response as waves III and IV of the acoustical response respectively.

In order to confirm that the electrical responses evoked in this way actually originated from auditory pathway activity the auditory nerve was stimulated electrically by two electrodes placed directly on the nerve. This procedure evoked the same pattern of response as elicited by electrical stimulation of the cochlea, though the latency was slightly shorter in direct nerve stimulation (Fig. 5). The vestibular nerve was also stimulated directly at the orifice of the internal auditory meatus (Fig. 6). This vestibular nerve stimulation evoked a phasic response which probably originated from vestibular nuclei. The shape of this response was completely different from those elicited by acoustic stimulation by electrical cochlear stimulation or by electrical auditory nerve stimulation. Facial nerve stimulation evoked no brain stem response by this procedure (Fig. 7). These results suggest that the electrical stimulation of the cochlea worked effectively as a stimulus to the auditory nerve.

COMMENT

In their recent paper Chouard et al. (1979) reported on BSER in man evoked by electrical stimulation of the round window. They supposed that this test was indispensable when considering surgical rehabilitation through a multichannel cochlear implant. By this test, it was possible objectively to ascertain if the functional auditory nerve fibres were still alive or not. They stated that the general features of BSER were similar enough in the case of electric or acoustic stimulation although the earlier waves of the electrical response might be absent because of the interference effect of the electric stimulus.



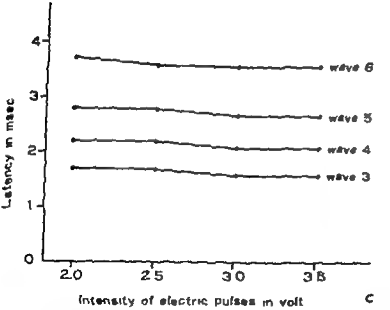
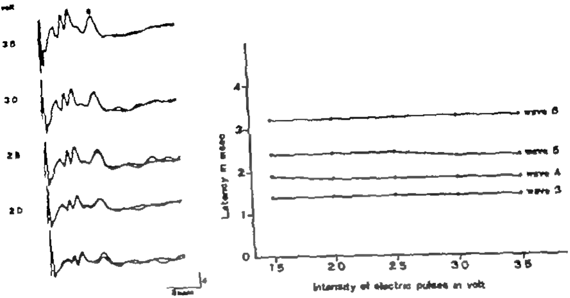


Fig. 4. BSERs by electrical stimulation of the cochlea. The electric stimulus was given through paired electrodes, one to the scala media of the third turn and the other to the scala tympani of the basal turn. The polarity of the electric pulse in the scala media was both (*a*) negative and (*b*) positive. There are no marked differences in the shapes and latencies of these responses. Waves 3, 4 and 6 are shown to be prominent and stable just as they are in auditory response. (*c*) Relationship between stimulus intensity and response latencies.

evoked by electrical stimulation of the cochlea through paired electrodes, one to the scala media of the third turn and the other to the scala tympani of the basal turn. This procedure evoked a response similar to the acoustic response, though the electrical response had a steeper waveform and the intervals between the waves were shortened. The stim



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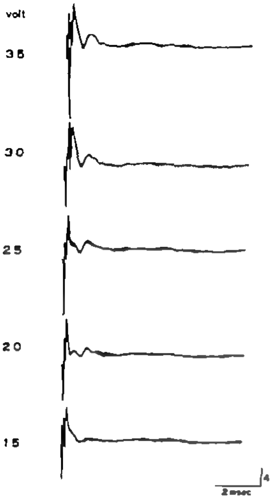

Fig. 6. BSERs by electrical stimulation of the vestibular nerve. Different from the responses in Figs. 4 and 5, vestibular nerve stimulation evoked a phasic response which lasted at most 1.5 msec.

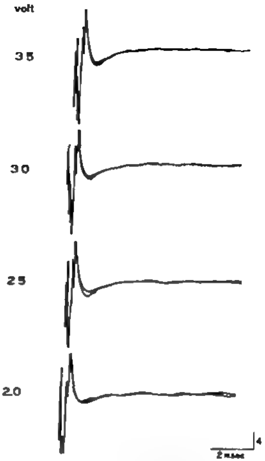

Fig. 7. Facial nerve stimulation evoked no recognizable brain stem response with this procedure.

Our present study using the guinea pig also showed that the shape of the BSER by electrical stimulation of the cochlea looked similar to that evoked by acoustic stimulation. The main difference was that the electrical response had a shorter latency and a steeper waveform, which perhaps reflected the more complete synchronization of the responses by electrical stimulation. Moreover, the shapes of the BSERs elicited by electrical stimulation of the cochlea and the auditory nerve were overwhelmingly similar, though the response latency of the latter was slightly shorter. This constituted direct proof that the electrical stimulation of the cochlea worked effectively as a stimulus to the auditory nerve. These relationships are presented in Fig. 8. When the cochlea was electrically stimulated, the recorded response might have been a mixture of vestibular and auditory ones. As the peak latency of the vestibular response was at most for 1.5 msec, waves 3 to 6 of the electrical response were only slightly affected by them or not at all. Thus, when these waves were chosen for study, we could distinguish the auditory activity from the response evoked by electrical stimulation of the cochlea without regard to the effect of the vestibular activity.

The advantage of recording BSER in a cochlear implant experiment lies mainly in long-term observation of the animal response. This method might be useful (1) to investigate with animals the most suitable electrode position for the cochlear implant, (2) to examine physiologically the damage of the cochlear sense organ by long term electric stimulation.

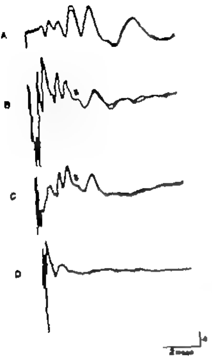

Fig. 8 Comparison of the acoustic and electric BSERs. These responses were evoked by (A) acoustic stimulation with click at 100 dB SPL, (B) electrical stimulation of the cochlea at 3.5 V scala media is negative (C) auditory nerve stimulation at 3.5 V and (D) vestibular nerve stimulation at 3.5 V

(3) to inspect how much the foreign-body reaction around the electrodes decreases the effective electric current flow These were not well examined through histological study However as BSER is not a frequency specific response frequency coding of the auditory sensation by electric stimuli cannot be evaluated by this method

Our present study shows the results of only a limited number of animal experiments This procedure might need further experimental study especially to clarify the significance of the electrically evoked brain stem response

ACKNOWLEDGEMENT

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ZUSAMMENFASSUNG

Die Hirnstamm-Reaktionen, die akustische und elektrische Reizungen auf Schnecken hervorriefen, wurden bei Meerschweinchen verglichen. Die elektrische Reizung wurde mit zwei Elektroden gegeben, eine Elektrode zur Scala tympani der basalen Drehung und die andere zum Schneckengang der dritten Drehung. Sechshundert fortlaufende Reaktionen wurden vom Computer summiert. Die Reaktionen, die verschiedene unmittelbare Reizungen auf dem N facialis, N vestibuli und N acusticus hervorriefen, wurden mit ähnlichen Verfahren für den Vergleich registriert. Die elektrische Reizung auf der Schnecke konnte die Reaktion mit fortlaufenden fünf oder sechs Wellen in weniger als 10 msec nach Reizbeginn hervorrufen. Diese hervorgerufene Reaktion war ähnlich der durch akustische Reizung hervorgebrachten. Die unmittelbare Reizung auf dem N acusticus rief auch eine ähnliche Reaktion hervor Die Reizungen auf den N vestibuli und N facialis bewirkten gänzlich verschiedene Reaktion oder riefen nicht hervor

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K afume Gyo M D
Department of Otolaryngology
School of Medicine
Ehime University
Shigenobu-cho
Onsen-gun Ehime
Japan 791-02

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THE INFLUENCE OF ULTRASOUND AND TEMPERATURE ON THE COCHLEAR MICROPHONIC RESPONSE FOLLOWING A ROUND WINDOW IRRADIATION

S B Barnett

From Ultrasonics Institute Sydney Australia

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Abstract Impairment of cochlear function was investigated following ultrasonic irradiation of the vestibule and the cochlea through the round window in cats and guinea pigs Selective destruction of the vestibular balance mechanism with negligible impairment of cochlear microphonic response was achieved provided that the ultrasound beam was directed away from the cochlea and towards the ampulla of the superior semicircular canal Directing ultrasound into the cochlea produced a depression in C M which was greatest in the higher frequency responsive area corresponding to the region of the first two cochlear turns The degree of cochlear microphonic depression increased as the duration of irradiation was extended The occurrence of a significant temperature increase accompanying the application of ultrasound implicated the involvement of a thermal mechanism in addition to the mechanical disruptive effect of ultrasound

Ultrasound has been applied to the vestibular labyrinth in the treatment of Meniere s disease since it was first introduced by Arslan (1964) Refinements in the operative technique and ultrasonic instrumentation led to the development of the round window approach by Kossoff et al (1967) in which the safety aspect was greatly improved with regard to avoiding facial nerve traumatization and hearing impairment. In the current investigation changes in the cochlear microphonic (C M) response were measured following irradiation through the round window either into the cochlea or towards the vestibule as in the treatment of Meniere s disease The influence of temperature increases which accompany continuous wave ultrasonic irradiation was also investigated to try to assess the relative importance of this mechanism in ultrasonically induced changes in the inner ear

Instrumentation

The round window ultrasonic applicator consists of a flat 3 5 MHz transducer 1 ? mm in diameter which is mounted in a cylindrical holder The maximum acoustic power output delivered from this transducer was 100 mW as measured by the radiation pressure balance technique being equivalent to an average intensity of 10 W/cm² Ultrasonic energy is emitted normal to the front face and coupled to the inner ear through a medium of distilled water

The experiments were undertaken with the animals under deep Nembutal (sodium pentobarbitone) and Alloferin anaesthesia. Respiration was maintained by a positive pressure respirator connected by a tracheotomy tube inserted at the level of the fourth tracheal ring The tympanic bulla was opened to display the entire cochlea and round window niche by means of a ventrolateral surgical approach Auditory stimuli over the frequency range of 500 Hz to 8 kHz were produced by a Bruel & Kjaer beat frequency oscillator and an Altec speaker The continuous tone was delivered at the tympanum through a conical module which also contained a ¼-inch probe tube and condenser microphone to monitor sound pressure level (S P L) of the stimulus The microphonic response was recorded with a micromanipulator mounted Ag/AgCl ball electrode on the round window membrane and a reference electrode placed on the neck muscles The stimulus and response signals were

Fig 1 Loss of postural control in cat no 9 one day after irradiation of the right vestibular labyrinth with 100 mW for 20 min

filtered and calibrated through a Brüel & Kjær audio frequency spectrometer and displayed on a Tektronix 503 oscilloscope

Irradiation methods

The first part of this investigation involved irradiating the vestibule through the round window Cats were selected as experimental subjects in preference to guinea pigs because the similarity of internal dimensions of their inner ear labyrinth allows propagation of ultrasonic energy by reflections within which more closely resembles that occurring in humans It was also found that the restricted size of the round window aperture and the anatomical displacement of the vestibule made it impossible to direct ultrasound reliably into the vestibule in guinea pigs During vestibular irradiation the ultrasound beam traverses a region of the basilar membrane approximately 2 mm in width and the effect of this on the microphonic response to stimuli within the range of normal speech frequencies was studied

In the second part of this study ultrasound was directed into the cochlea through the round window in guinea pigs and cats The effect on C M was determined following insonation with 100 mW for durations of 5 to 20 min. In addition in 3 non-irradiated cats the round window membrane was deliberately damaged by driving the ultrasound probe through it until a rupture was produced The effect on the microphonic response of this lesion was compared with changes occurring following cochlear irradiation

In a final series of experiments ultrasonically induced temperature changes were measured within the inner ear with implanted calibrated miniature bead thermistors while the vestibule and the cochlea were irradiated through the round window with 50 and 100 mW The influence of this temperature rise on cochlear function was then investigated by increasing the intracochlear fluid temperature by an amount which exceeded that measured during the irradiation and recording changes in the microphonic response A pilot study with similarly implanted thermistors had previously established that adequate thermal conduction could be achieved throughout the cochlea by irrigating the tympanic bulla with preheated water This method was then used to determine the effect of such a temperature rise on C M in the absence of ultrasound The microphonic response was recorded during 5 min of this treatment and for 5 min thereafter

RESULTS

Vestibular irradiation

Vestibular ablation was confirmed from observations of balance dysfunction during the post-irradiation recovery period Cats typically displayed paralytic nystagmus a severe leaning of the head towards the operated side and loss of balance and locomotory control causing them to fall onto the irradiated side (Fig. 1) Cats not displaying these symptoms were considered to be incorrectly irradiated and microphonic data obtained from them was discarded

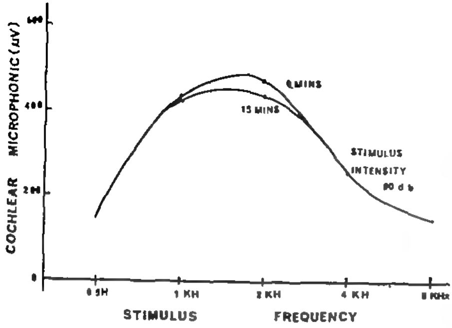


Fig 2 The frequency related cochlear microphonic response is illustrated following ultrasonic irradiation. A negligible change followed 100 mW vestibular irradiation for durations up to 15 min

Irradiation through the round window resulted in a negligible change in the cochlear microphonic response to stimulus frequencies ranging from 500 Hz to 8 kHz (Fig 2) Assuming that similar propagation of ultrasound energy occurs within the inner ear labyrinth of humans the treatment of Meniere s disease would have no detrimental effect on hearing within the range of normal speech frequencies

Cochlear irradiation

Irradiating the cochlea through the round window with 100 mW resulted in a depression in the microphonic response over the range of stimulus frequencies The occurrence of a significant (75%) reduction in response to 4 kHz indicates that ultrasonically induced damage occurred at least as far as the second cochlear turn which is responsive to this frequency Whilst accepting the limitations of round window C M recordings (Dallos et al 1971) it was observed that significant changes occurred in response to frequencies as low as 500 Hz In addition histological examination has confirmed the occurrence of pathological changes extending into the third turn following round window irradiation with a similar ultrasound regime (Barnett 1979) In the present study the degree of C M depression was also found to increase as the duration of irradiation was extended from 5 to 20 min (Fig 3)

Temperature measurement

During an irradiation the greatest temperature increase was measured at the transducer face and in the round window niche and is due to the influence of self heating The degree of heating is dependent on the efficiency of the transducer and may account for the variability in temperature changes reported elsewhere following irradiation of the inner ear labyrinth (James & Halliwell 1970) A rapid rise in temperature occurred in the proximal region of the cochlea the magnitude of which was related to the average acoustic power level (Table I) Irradiation with 100 mW resulted in an 8°C temperature increase in the basal turn after 3 min (Fig 4)

Thermal effect

Increasing the intracochlear fluid temperature by 5°C above normal body temperature had no effect on the microphonics at the round window whereas a 7.5°C elevation resulted in a reduction of C M by approximately 75% after 2½ min (Fig 5) Raising the temperature by 10°C produced an 87% reduction in C M within 1½ min During the series of experi-

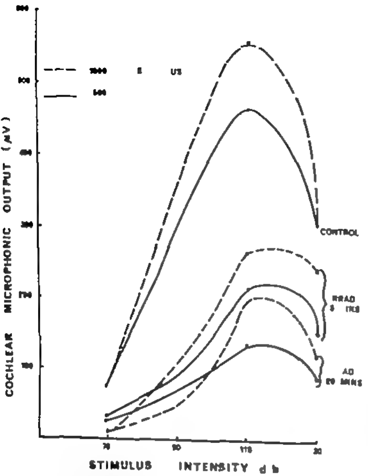


Fig. 5 The microphonic response measured at the round window following cochlear irradiation with 100 mW for 5 min and 20 min, showing progressive decrement with increasing duration of irradiation.

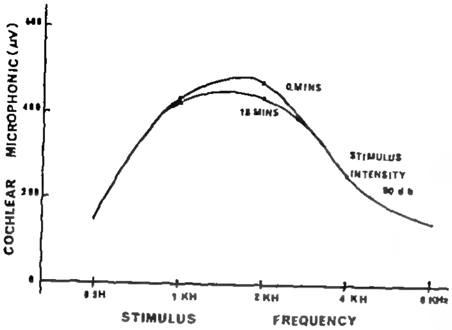
ments it was found that the threshold for C M impairment was around 7°C above normal and that when temperatures just above this threshold were applied for short periods of 30 sec or less a reduction of 15–20% in C M occurred which was completely reversible. However when a suprathreshold temperature was applied for more than 1–2 min a permanent depression in C M occurred. At temperature elevations of 7.5° and 10°C C M was reduced in response to all frequencies.

Table I *Maximum temperature increases recorded after 3 min of continuous irradiation with time-averaged acoustic power levels of 90 to 100 mW applied into the cochlea through the round window*

Thermistor location	Maximum temperature increase	
	90 mW	100 mW
Round window niche	5 8	1..3
Cochlea turn 1	3 4	8 4
Cochle turn 2	2 9	6.1
Cochlea turn 3	9	3 9
Cochlea turn 4	.5	3.8
Facial nerv	0 8	1 6

Ruptured round window membrane

Experimental rupturing of the round window membrane resulted in practically total abolition of the cochlear microphonic response over the range of stimulus frequencies used (Fig. 6). The disruption in ionic balance between the endolymph and perilymph resulting from this lesion effectively cancelled cochlear function throughout.



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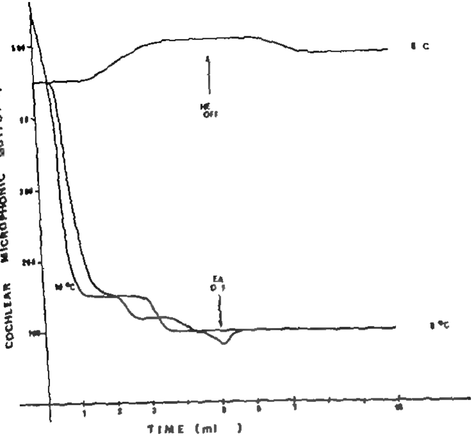

Fig. 5. The effect of elevating the intracochlear temperature on the cochlear microphonic response showing that increases of 7.5°C and higher result in permanent impairment of hair cell function.

threshold for lesion production is temperature dependent (Dunn & Fry 1971) such an increase in temperature may also increase the extent of damage accompanying the fixed intensity of 100 mW

Using a large diameter transducer Seda et al (1971) irradiated the vestibule and part of the basal turn of the cochlea through the bone with 700 mW for 20 min. The reported reduction in C M response over the range of frequencies from 500 Hz to 10 kHz was probably due to the large temperature increase generated by absorption of ultrasound into the 0 5 mm thick osseous wall

When temperature was measured during irradiation the rapid increase occurring in the first two cochlear turns indicates that this is the minimum extent of transmission of ultrasonic energy from the round window The occurrence of a similar rapid temperature increase within the vestibule may cause convection currents and initiate the irritative nystagmus typically observed at the beginning of the ultrasonic treatment of Meniere s disease Slower increases in remote regions of the cochlea probably resulted from conductive heating throughout the intracochlear fluid

The results of the thermal influence indicate a damage threshold level of 7.5°C temperature increase above which an irreversible depression in the microphonic response occurs. Irradiation with 100 mW raised the intracochlear temperature by 8 0°C in the basal turn, and as this exceeds the temperature threshold the

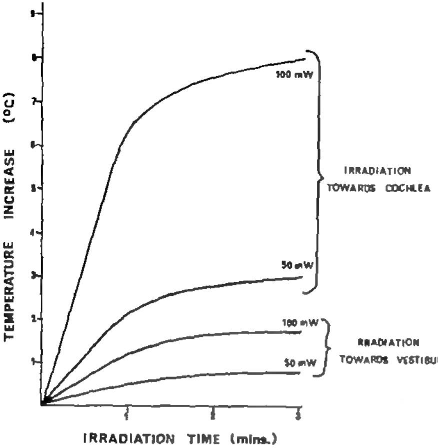

Fig 4 The effect of ultrasound on intracochlear temperature in the basal turn of the cochlea related to the direction of irradiation.

DISCUSSION

During vestibular irradiation the direction of application of the ultrasound beam was found to be critical and deviations from the correct course produced minimal vestibular response. The most severe reaction was observed following the application of ultrasound towards the position of the ampulla of the superior semicircular canal. This finding is relevant to the clinical treatment of Meniere's disease and suggests that optimal results will only be achieved when the ultrasound beam is accurately directed in this manner. The absence of either nystagmus or cristae damage reported by Bouchard & Benitez (1978) following a similar round window irradiation may be due to differences in direction of irradiation within the vestibule. The minimal impairment of cochlear function confirms the safety aspect of the round window treatment of Meniere's disease. The earlier report of Kossoff (1972) is reiterated by these results where the level of acoustic power was doubled. The results are also in agreement with the audiometric findings of the majority of patients in a long-term clinical evaluation of the treatment (Barnett & Kossoff 1977). Although postoperative hearing losses have occasionally been detected, the audiograms typically show a flat loss consistent with the effect of experimental round window rupture on C M (Fig 6). Such hearing losses would seem to have resulted from surgical damage rather than the influence of ultrasonic energy within the cochlea which would produce a preferentially high frequency loss.

Cochlear irradiation resulted in a severe depression of C M which demonstrates the importance of accurately aiming the ultrasound beam in order to avoid intracochlear damage during Meniere's treatments. The progressive reduction in C M which occurs as duration of irradiation is increased beyond 5 min (Fig 3) may result from the temperature build up of 8°C which accompanies irradiation into the cochlea. As the intensity

die Vestibularisfunktion selektiv zerstört, während die Einwirkung auf die Cochlearmikrophone-Response vernachlässigbar war. Ultraschall-Beschallung der Cochlea hatte eine Abnahme der Cochlearmikrophonie zur Folge und zwar am stärksten für höhere Frequenzen und in zunehmendem Maße mit wachsender Dauer der Beschallung. Unter der Beschallung wurde in der Basalwindung eine Temperaturzunahme von 8°C gemessen, die damit die Temperatur überstieg, bei der die Veränderung der C.M. irreversibel wird. Das bedeutet, daß zusätzlich zu der zerstörenden mechanischen Wirkung des Ultraschalls auch ein thermischer Mechanismus einbegriffen ist.

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S. B. Barnett, B.Sc., M.Sc.
Ultrasonics Institute
5 Hickson Road
Sydney, N.S.W. 2000
Australia

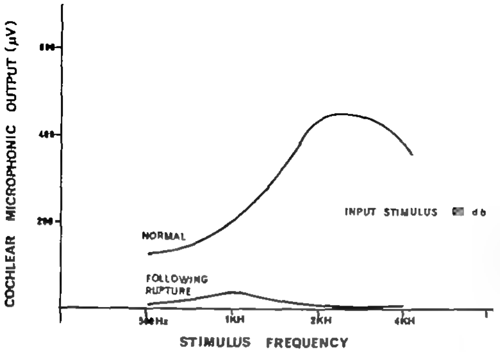

Fig 6 The experimental rupturing of the round window membrane resulted in a 'flat' depression in cochlear microphonic response

thermal influence alone would be expected to significantly affect C M The 6°C increase recorded in the second cochlear turn should not affect the microphonic response though results have shown depressions in C M in addition to hair cell disruption (Barnett 1979) throughout the first two cochlear turns following ultrasonic irradiation As conductive heating from the round window niche would produce a subthreshold increase this must be excluded as a cause of C M depression suggesting that the deleterious effects are due to another mechanism i e ultrasonic agitation The situation is complicated by the fact that temperature increases recorded during irradiation represent the average of the intracochlear fluids The increase would be higher in tissue lying in close proximity to the bony periosteum due to large absorption of the ultrasonic energy by bone This local heating may be sufficient to cause local cellular disruption before being dissipated There seems to be an involvement of a thermal mechanism in addition to the mechanical agitating effect of ultrasound when C M is depressed during ultrasonic irradiation

During experiments in which the irradiation treatment mimicked the procedure for Meniere s disease therapy a temperature increase of 7°C was measured within the vestibule which is below the temperature damage threshold for neuro-epithelial tissue This suggests that the destruction of the otoliths results from the influence of ultrasound rather than conductive heating Meanwhile the intracochlear temperature was raised by only 1 7°C when 100 mW was correctly aimed at the vestibule Being well below the threshold level this is further endorsement of the safety aspect of this form of clinical treatment

SUMMARY

Vestibular balance function was selectively destroyed whilst negligibly affecting the cochlear microphonic response when ultrasound was directed through the round window toward the ampulla of the superior semicircular canal Cochlear irradiation produced a depression in C M which was greatest at higher frequencies and increased when the duration of irradiation was extended The accompanying temperature increase of 8°C measured in the basal turn exceeded that required t reduce C M reversibly and implicated the invol ement of thermal mechanism in addition to the disrupti e effect of ultrasonic agitation

ZUSAMMENFASSUNG

Durch Ultraschall-Beschallung durch das runde Fenster gegen die Ampulla des oberen Bogenganges hin wurde

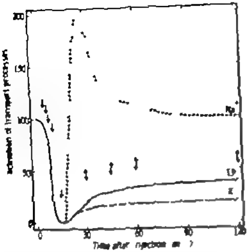

Fig. 1. The commonest time course of the action of L ethacrynic acid (60 mg kg^{-1}) upon the strial transport mechanisms found by Bosher (1980). Morphological examination of animals conforming to this pattern was undertaken at the points indicated by the arrows; the actual number in each instance is shown in Table I. The degree of activation of the mechanisms is expressed relative to the normal level (100%).

METHODS

Young Sprague Dawley albino rats, who had not been exposed to infection or antibiotics, were used. The anaesthesia, animal preparation and electrophysiological procedures are described in the earlier paper (Bosher, 1980), which gives a full account of the functional derangements produced by a single injection of ethacrynic acid (60 mg kg^{-1}) into the right femoral vein.

A difficulty arose because of the small variations in the time course of the drug's effects upon the endolymph system. In order to standardize the experimental conditions, therefore, only animals whose changes corresponded to the time course of the inhibition of the strial transport processes shown in Fig. 1 were selected for anatomical examination. This was the commonest time course found and the procedure eliminated the quite marked differences in the extent of the ultrastructural abnormalities found at 120 min in the preliminary study (Bosher et al., 1973). The times

Table I. *The distribution of the experimental material*

Time after injection (min)	5	7	8	10	15	30	45	60	120
N of animals examined	3	1	1	6	4	2	2		2

judged to provide a comprehensive picture of the strial changes, together with the number of subjects examined at each time, are given in Table I. Six control animals, injected with saline, were also examined.

The animals were decapitated, the temporal bones removed and the cochleae fixed initially by perfusion of the perilymphatic spaces through the oval and round windows (a small opening having been made at the apex) with phosphate buffered (pH 7.2–7.4) 1% osmium tetroxide at 4°C. Fixation was completed by immersion of the cochleae in the same fixative for 2 hours. Specimens were embedded in Epon and divided into appropriate segments by means of a jeweller's lathe. Thin sections were prepared using a Reichert OM3 ultramicrotome, stained with uranyl acetate and lead citrate, and examined in a Philips EM 300 electron microscope.

Unfortunately, some aberration in the polymerization of the Epon in the two 45-min animals rendered it electron-dense. The material was not examined for about a year after its preparation, when it was discovered that variations in the size of the interstitial spaces which had not been described previously occurred during the course of the recovery period. Now, the preliminary investigation (Bosher et al., 1973) had shown the size of these spaces to be readily affected by the experimental circumstances. For the sake of scientific veracity, therefore, it was decided to present the faulty material rather than prepare new specimens, because all the results (both anatomical and physiological) would then come from the same group of animals. The author apologizes for the consequent poor quality of Fig. 6A.

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THE NATURE OF THE OTOTOXIC ACTIONS OF ETHACRYNIC ACID UPON THE MAMMALIAN ENDOLYMPH SYSTEM

II *Structural–Functional Correlates in the Stria Vascularis*

S K Bosher

From the Ferens Institute of Otolaryngology The Middlesex Hospital Medical School University of London London England

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Abstract The relationship of the ultrastructural changes in the stria vascularis to the functional alterations in the endolymph system was investigated in 23 rats at various times up to 170 min after a single intravenous injection of ethacrynic acid (60 mg kg) The overall effect upon the marginal cells revealed by the development and regression of marked cytoplasmic swelling, was reasonably well correlated with the derangements produced in the strial ion-transporting mechanisms. The pathophysiological relationships of the action upon the intermediate cells was less exact and no unequivocal structural correlates were found for the diminution in membrane permeability and the probable late decrease in energy production. The size of the interstitial spaces was determined mainly by the variations in volume of the marginal and intermediate cells

A number of accounts of the ultrastructural abnormalities produced in the stria vascularis by the ototoxic diuretic ethacrynic acid have appeared since the initial paper by Quick & Duvall (1970) However two deficiencies have become apparent in our knowledge of this subject Firstly it is not certain to what extent a correlation exists between the specific anatomical changes described and the complex functional alterations now known to occur largely because the associated physiological investigations have been of a restricted phenomenological nature Secondly the time intervals selected have been large and varied so that it is not clear whether the different features reported arise from the divergences in the experimental circumstances or whether they could represent successive phases in the same basic process

Recently Brummett et al (1977) have provided further valuable information about these aspects in their wide ranging study of the effects of i v ethacrynic acid in the guinea pig. The morphological derangements present in their material suggested that the condition of the apical ends of the marginal cells was more closely related to the action upon the endocochlear potential (measured with the cochlear ac potential in the same animals) than were the other strial changes during the acute phase Nevertheless even these authors only examined the stria vascularis at 30 48 108 and 230 min and their encouraging findings re emphasize the need for a more intensive approach

A detailed investigation into the physiological effects of i v ethacrynic acid upon the endolymph system in the rat (Bosher 1980) provided a good opportunity to determine the sequence of the anatomical alterations more precisely and to relate them to the functional changes Animals were examined at more frequent intervals than before between 5 and 170 min after administration of the drug The time courses of the ultrastructural derangements in the marginal cells and the action upon the strial transport processes corresponded closely but a simple correlate was not found for either the morphological abnormalities induced in the intermediate cells or the enlargement of the interstitial spaces the feature stressed most often in the past

RESULTS

Control animals

The general structure of the stria vascularis and the detailed morphology of its constituent marginal intermediate and basal cells are virtually identical in the rat to the now classical descriptions in other species (Engström et al 1955 Hinojosa & Rodriguez Echandia, 1966 Smith 1957 Spoendlin 1967) The only feature calling for further comment is the identity of the vesicular structures present in the apical regions of the marginal cells

Most of the vesicles have an internal surface coat and so are members of the lined group (Brummett et al 1977) but they seem to be of varied origins and at least three general types can be distinguished The first type is usually present in large numbers and is much smaller than the others having a diameter of approximately 70 nm These vesicular elements are associated with occasional short tubules of the same size and configuration (Fig. 2) The character and distribution of this group of structures indicates that they belong to the apical tubular network of smooth endoplasmic reticulum which is now thought to be a universal feature of ion-transporting epithelial cells (Møllgård & Rostgaard 1978) Typically the network is orientated in parallel with the luminal surface and is thus seen as separate vesicles in perpendicular sections a situation responsible for its delayed recognition

The vesicles in the second group are less numerous more irregularly distributed and bigger in volume being about 0.5 μm in diameter Their size and appearance is identical with the so-called pinocytotic vesicles budding from (or merging with) the surface membrane The third group is much rarer and consists of isolated large vesicles (0.8 μm or more in diameter) and nothing of certainty is known about their origin True bristle-coated vesicles are also present in small numbers as are other solitary vesicles some of which are related to the Golgi complexes

Examination of the 6 control animals revealed no evidence of any abnormality resulting from the injection of saline

Development of the ultrastructural alterations

Of the 5 animals examined 5–8 min after the injection of ethacrynic acid, 3 were completely normal The other two both 5-min animals had developed very slight changes representing the initial morphological effect of the drug.

A variable degree of cytoplasmic shrinkage was present in some intermediate cell processes and bodies As a result, localized collections of fluid had formed in the interstitial spaces (Fig 3A B) The organelles and the remainder of the intermediate cells appeared quite unaffected and no alterations were visible in the marginal cells, the only other abnormality involving the capillary basement membrane The peripheral portion of the membrane related to the marginal and intermediate cell processes, had lost its usual dark character over large areas (Fig. 3C) Whether the membrane involved had disappeared or had merely lost its staining properties could not be determined.

The most striking aspect of this initial stage was the scattered distribution of the lesions. In any one section only a small number of cells and capillaries were abnormal No particular site was predominantly involved although the intermediate cell processes near the capillaries were affected more frequently than those elsewhere Furthermore the stria vascularis was entirely normal at many points along the cochlea. The pathological changes could be found in any area and the more apical portions were not selectively spared The greater susceptibility of the few damaged cells was therefore not related to their position within the cochlea unlike the effect after subcutaneous intraperitoneal and oral administration (Anniko 1978 Forge 1979b Johnsson & Hawkins, 1972)

At 10 min all 6 rats were affected. In one the appearances were the same as those just described with scattered early lesions sepa

F g 2 Normal marginal cell with elements of the vesiculo-tubular system of smooth endoplasmic reticulum (*arrows*) The usual Golgi membranes rough endoplasmic reticulum free ribosomes and glycogen granules, together with a pinocytotic vesicle ( ) and a mitochondrium are present A thin surface coat s visible Bar 1 μm

Fig. 4. Moderately severe changes at 10 min. *A*. Swollen marginal cell in the absence of interstitial spaces or intermediate cell involvement. Bar 4 μm. *B*. Swollen marginal cells associated with moderate interstitial spaces and shrunken intermediate cell. Note the breaking up of the parallel membranes of the deep processes (centre) and the localised bulging on one cell (arrow). The space between the basal cells ( ) is atypical. Bar 4 μm. *C*. Marginal cell showing normal cytoplasmic organelles, except for absent micropinocytotic vesicles. Bar μm. *D*. Severely shrunken intermediate cell. Note the normal organelles and the persistence of its contact with the capillary (bottom). Bar μm.

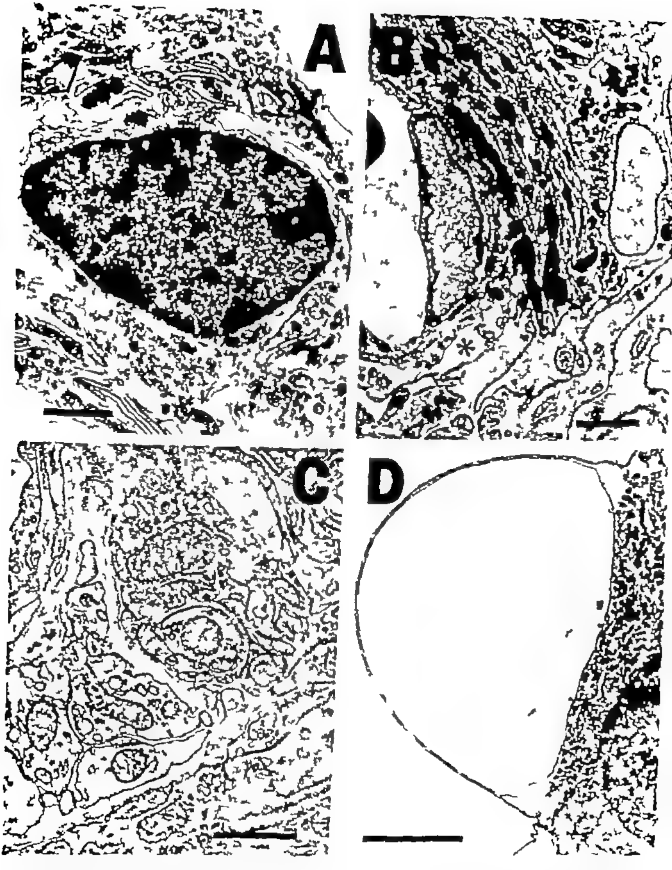


Fig 3 Early changes. *A* Localized area of shrinkage of intermediate cell body (*arrow*) at 10 min Bar 3 μm *B* Isolated shrinkage of intermediate cell process with corresponding collection of interstitial fluid ( ) at 5 min An adjacent intermediate cell is normal Bar 3 μm. *C* Increased translucency of the outer leaflet of a capillary basement membrane at 5 mi Bar 1 μm *D* An enormous membrane-bound vesicle i a marginal cell at 15 min Similar structures were found on only two other occasions and its presence may be fort itous Bar 1 μm

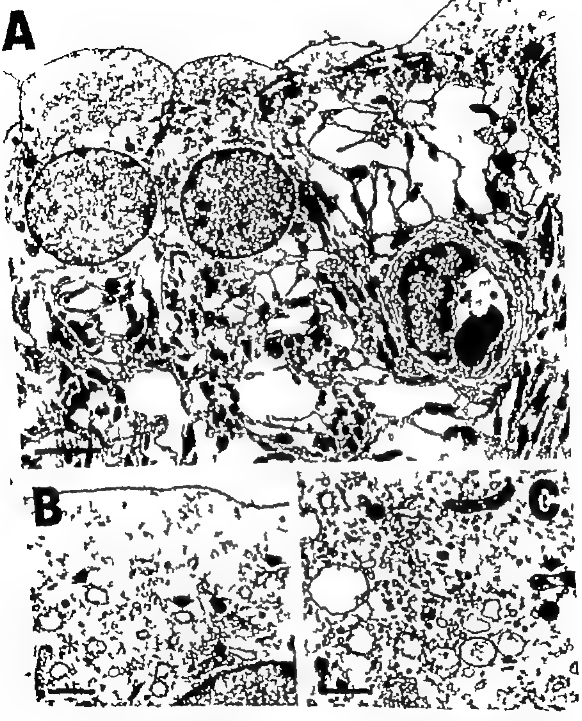


Fig. Maximally severe changes at 30 min. *A.* General view with apposition of the endolymphatic surfaces burying the unaffected tight junctions (*upper left*). Bar 3 μm. *B.* Marginal cell demonstrating the enlarged rough endoplasmic reticulum (arrow). T abnormal vesicles are present ( ). Bar 1 μm. *C.* Marginal cell to show the normal vesiculo-tubular elements. Several pathological vesicles are included and mitochondrion exhibits small vacuolated area (arrow). Bar 1 μm.

rated by intervening normal portions But in the 5 remaining animals the stria vascularis was abnormal in all areas more severely involved regions intermingling haphazardly along the length of the cochlea with those showing only early alterations

The most significant change where the intoxication had progressed was slight to moderate cytoplasmic swelling of the marginal cells which was confined to the cell body the superficial cytoplasm often being most affected without noticeable involvement of the deep processes (Fig 4A B C) It caused a corresponding bulging of the apical membrane into the endolymphatic space This could be irregular and in a few instances was limited to a part of the cell surface (Fig. 4B) as described by Forge (1979*b*) There was no close correlation with the intermediate cell alterations the marginal cells occasionally being involved alone (Fig 4A)

Their surface microvilli disappeared during the initial stage of the swelling and subsequently no micropinocytotic vesicles were present either at the surface or deeper in the cytoplasm Otherwise all the intracellular organelles appeared entirely normal including the smaller vesiculo-tubular elements the rough endoplasmic reticulum the Golgi apparatus and the mitochondria They tended to become dispersed due to the swelling leading to some reduction in density but not in number The swollen cytoplasm was also less electron-dense than usual (Fig 4A B C)

The only other marginal cell component to be affected was the plasma membrane of the deep processes Santos-Sacchi (1978*b*) has very ably described the rows of small vesicles produced in place of the parallel membranes lining the deep interdigitating clefts by osmium fixation Areas of membrane exhibiting such artifactual alterations normally intermingle with well preserved plasma membrane infoldings and probably indicate sites of differing membrane activity In the swollen marginal cells the tendency for these plasma membranes to break down during fixation was greatly accentuated and parallel membran could be entirely absent (Fig 4B) This fe ture strongly suggested that the ethacryn acid had brought about changes in the mem branes of the deep processes thus adding the evidence provided by the clumping of n tramembrane particles seen in freeze fractu preparations (Forge 1979*a*) The cytoplasm microtubules described in the deep processe disappear during osmium fixation (Santos Sacchi 1978*a*) so their state could not be as sessed in the present investigation

Although normal intermediate cells could b identified (Fig 4A) they were rare for n general they had become shrunken con comitant with the marginal cell involvemen (Fig 4B) Nevertheless even when thr shrinkage was extreme the intracellular or ganelles showed no signs of abnormality and the intermediate cells maintained their contacts with the capillaries whose basement membranes had largely lost their translucent areas (Fig 4D)

At the same time there was a corresponding increase in the size and number of the interstitial spaces which were clearly evident scattered throughout the stria, but the tight junctions between the marginal cells were unaffected However it was noteworthy that no spaces were present where the intermediate cells were normal (Fig 4A) that they were not usually found in the clusters of marginal cell deep processes (Fig 4B) and that it was atypical for them to occur between the basal cells (Fig 4B)

At 15 min the intoxication had progressed and many areas scattered along the cochlea were maximally affected in all 4 animals but there were intervening less severely involved regions By 30 min however maximal changes were present in every part of the stria (Fig 5)

Here the swelling of the marginal cells had increased still further so that in many instances the adjacent surface membranes were pressed together for some distance above the buried tight junctions The swelling continued

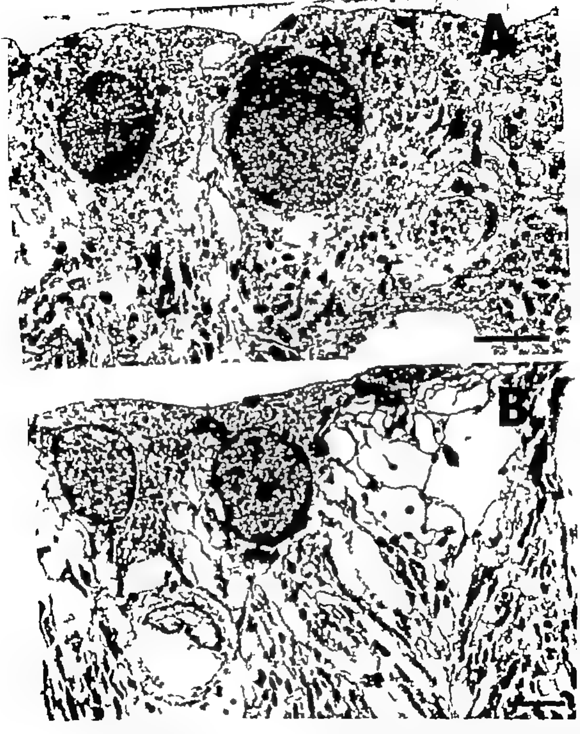


Fig. Recovery at 45 min (A) and 60 min (B). Bars, μm 4. The marginal cell swelling is reduced but the rough endoplasmic reticulum is still dilated (arrow). The interstitial spaces are smaller. (With apologies for the poor quality. See Methods.) *B*. The marginal cells [illegible] now slightly shrunken. All organelles are normal [illegible] pathological vesicles (arrow) uncommon. The interstitial spaces have become much larger.

to be confined largely to the superficial portion of the cell while the cytoplasm did not entirely lose its electron-dense staining character except where rare vacuolated' (not membrane bound) clear areas could be found The rough endoplasmic reticulum had become dilated with relatively dark staining contents (Fig 5B) In a few cells occasional mitochondria had developed small vacuolated areas (Fig 5C) but the remainder looked normal as did the other organelles Pathologically large vesicles often containing a number of small vesiculated bodies were common even if irregularly distributed In addition the alteration in the character of the membranes of the deep processes continued unchanged

Almost all the intermediate cells were grossly shrunken with more electron-dense cytoplasm than usual although the organelles again showed no evidence of damage The interstitial spaces were now very extensive (Fig 5A) but the tight junctions were not separated The capillary basement membranes had returned to normal

Recovery of the ultrastructural alterations

An early and comparable degree of recovery was present in both 45-min animals (Fig 6A) The swelling of the marginal cells was much reduced their endolymphatic surfaces typically being irregular although there was little change in the organelles with the continued dilation of the rough endoplasmic reticulum and the continued presence of the pathological vesicles All mitochondria now appeared normal but the membranes of the deep processes were still broken up during the fixation The intermediate cells were less shrunken and the interstitial spaces noticeably less extensive some regions showing greater improvement than others

By 60 min recovery had progressed again to the same extent in both the animals studied (Fig 6B) However the appearances were rather puzzling initially until close examination revealed that the marginal cell swelling had completely disappeared in every case Instead most cells were somewhat shrunken as indicated by the frequent contraction of the infranuclear cytoplasm to a narrow rim by the increased narrowness of the deep extensions and by the common presence of spaces within the clusters of deep processes for the first time The cytoplasm was also a little more electron-dense than usual A few surface microvilli had returned and micropinocytotic vesicles were present if scarce while the rough endoplasmic reticulum and the other organelles were now uniformly normal Pathological vesicles were extremely rare and the fixation artifact involving the deep processes was less widespread

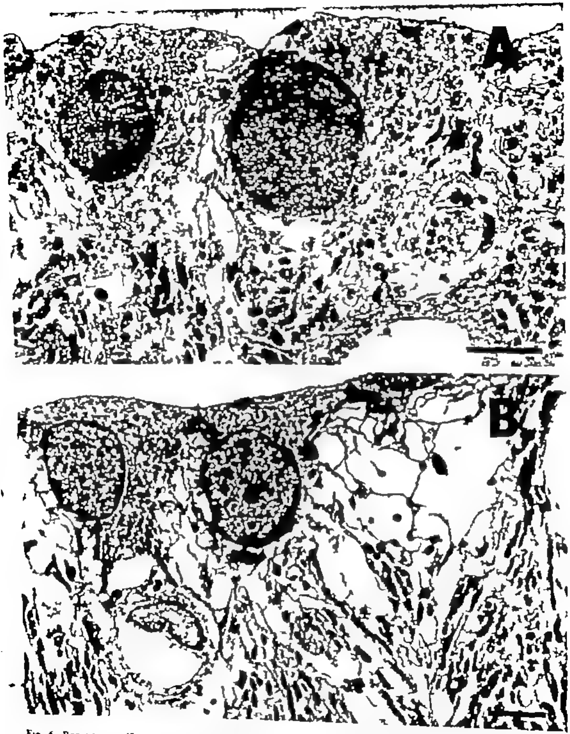
Careful assessment indicated that the intermediate cells had continued to increase in size and that the obvious expansion of the interstitial spaces which seemed so contradictory at first sight was the result of the marginal cell changes The intermediate cell organelles continued unaffected except for a suggestion of a rise in the number of Golgi complexes which was difficult to quantify

Finally in the 2 animals examined at 120 min full recovery had occurred apart from the persistence of a few residual irregularly distributed interstitial spaces In addition a number of small vesicles of uncertain origin and significance had appeared and were scattered throughout the marginal cells They could have been dilated members of the vesiculo-tubular system of smooth endoplasmic reticulum but this could not be established with certainty A subsidiary investigation of one 4-hour and two 6-hour rats revealed some interstitial spaces and the small vesicles still to be present at 4 hours but they had entirely disappeared at 6 hours

DISCUSSION

General considerations

The gross changes observed in the rat stria vascularis have been described in isolation by earlier workers but it has now become clear due to the smaller time intervals chosen



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that they constitute a progressive sequence of events in a single process rather than reflecting the differences in the species and procedures employed Perhaps the only two new findings at this level are the fluctuation which occurs in the size of the interstitial spaces and possibly the reversible generalized swelling of the marginal cells However when the details of the intracellular alterations are compared with the previous accounts (Anniko 1978 Brummett et al 1977 Johnsson & Hawkins 1972 Kasajima 1973 Nakai 1971 Quick & Duvall 1970 Silverstein & Yules 1971) it is immediately obvious that considerable variations exist both amongst the past investigations and between them and the study reported here These divergences almost certainly arise from the differences in the techniques doses and species selected even if their origin cannot be specified yet Detailed extrapolation of the results therefore seems inadmissible although the basic pattern of the pathological process is undoubtedly common to all

A general feature of the present experiments was the slight heterogeneity during the development of the intoxication some strial areas being affected earlier and to a greater extent than others Such a variation in cell susceptibility could reflect a similar variation in their physiological condition at the time the drug was administered If this was the case the question immediately arises whether the strial cells pass through successive phases of activity or whether the functional state of a cell is invariant and differs permanently in some way from its neighbours However other possible causes of the phenomenon cannot be ignored such as fluctuations in the metabolic supply or in the access of the drug due to differences in the geometrical relationship of the cells concerned to their blood supply In fact the observation again emphasizes how little is known about the complexities of strial function

The early inter animal variation could have a more prosaic explanation It is not easy at 5 or 10 min to decide exactly when the minimum peak in the endocochlear potential will occur and some lack of accuracy in predicting the time course is likely Nevertheless why animals should respond at different rates making the selection of a particular time course necessary is again obscure

The marginal cells

The outstanding change produced in the marginal cells was the cytoplasmic swelling resulting in the protrusion of their endolymphatic surfaces which has been so well demonstrated by scanning electronmicroscopy (Arnold et al 1978 Forge 1979*b* Horn et al 1977). There has been a tendency to attribute this protrusion to the effects of increased hydrostatic pressure arising from the accumulation of interstitial fluid However such an action can be excluded certainly in the present study for a number of reasons

Firstly the tight junctions are not tethered to the deep layers and if the marginal cell were simply forced into the scala media the tight junctions should also move in a similar fashion rather than become buried beneath the contiguous surface membranes Secondly there was no evidence of displacement of the deep processes towards the surface which would be unavoidable if the whole cell was undergoing protrusion Thirdly no correlation existed between the doming of the marginal cells and the volume of the interstitial fluid the marginal cells could be involved alone or could be normal despite gross expansion of the extracellular spaces (depending on the stage) And fourthly the general character of the alterations clearly indicated cytoplasmic swelling with consequent reduction in the density of staining

That such swelling should occur is really not surprising The control of cell volume depends on a variety of active and passive mechanisms which have been reviewed recently by Macknight & Leaf (1977) In almost all tissues which have been investigated under normal physiological circumstances ethacrynic acid causes cellular swelling associated with

an increase in the intracellular sodium concentration although the exact mechanisms underlying these changes are still the subject of debate Furthermore when recovery occurs under these conditions over-compensation is now known to take place with consequent shrinkage of the previously swollen cells to their so-called minimum volume which is about 0 8 normal (Kregenow 1977)

In the results reported here the degree of swelling of the marginal cells thus provides a good index of the extent of their intoxication by ethacrynic acid. Comparison with the functional effects described in an earlier paper (Bosher 1980) shows that a good correlation exists between this morphological abnormality and the inhibition of the strial transporting processes (Fig. 1) It is true that the swelling did not appear for 10 min while the latent period for the functional effects was about 2.5 min but this slight lag is well within the period to be expected for such structural alterations to develop (Macknight & Leaf 1977) Thereafter the swelling increased to a maximum in company with the similar increase in the ion-transport inhibition.

Next there was another short lag between the functional and structural changes at the onset of recovery which can be explained on the same basis as the earlier one But once it had commenced recovery was initially rapid in both respects and the slight shrinkage of the marginal cells showed over-compensation of the cell volume increase to have occurred at 60 min

Subsequently the marginal cell volume remained normal despite the reduction in the rate of recovery of the endolymphatic ion-transporting mechanisms presumably because the activity of these mechanisms was now sufficient to maintain the normal volume The reason why the cell body was predominantly involved is not known, it may indicate some physiological difference in the membranes of the deep processes but it may merely reflect their greater resistance to stretch

Associated with the increased swelling, was loss of the usual pinocytotic activity in the cell endolymphatic membrane While the phenomenon may have arisen from increased stretch of the membrane other causes cannot be excluded including a direct action of the ethacrynic acid Apart from this it was striking that all the other intracellular organelles remained normal at first Later the rough endoplasmic reticulum became selectively dilated and such a change might have been due to alterations in the intracellular composition (Clvan 1978) However the interrelationship of the organelles and volume regulation is not well understood at present (Macknight & Leaf 1977) and a more specific aetiology might have been present.

Even in the later stages most of the remaining organelles were still unaffected, so there could have been no major disruption of the cell and this must have been an important factor in its rapid and complete morphological recovery The small vesicles which became evident during this recovery seem likely to represent some compensatory reaction but nothing of certainty is known about them or about the origin of the pathological vesicles which might be related either to the volume derangements or to the ethacrynate intoxication.

The mitochondrial abnormalities are difficult to assess. The severe reduction in mitochondrial activity produced by ethacrynic acid in isolated renal mitochondria is not associated with any obvious structural derangement (Case et al. 1973) so where this does occur the functional effects must have been gross In the present study there was almost certainly a decrease in the mitochondrial energy supply at about 25–30 min and the occasional mitochondrial vacuolation seen at this time probably represents the action upon the most vulnerable of these organelles Support for this view is provided by the similar but more extensive changes reported in the guinea pig (Anniko 1978 Brummett et al 1977).

The intermediate cells and interstitial spaces

It does not seem to be generally appreciated that the marked shrinkage of the intermediate cells represents a most unusual and possibly unique reaction to ethacrynic acid intoxication (Macknight & Leaf 1977) The identity of the cellular functions responsible for it are obviously vital to our understanding of the cell's role Yet our knowledge is too meagre even for the most tentative of hypotheses In addition the shrinkage must be accompanied by disruption of any intercellular communications (Jahnke 1975 Reale et al 1975) although the importance of this is again unknown The lack of any effect upon the intracellular organelles contrasts with the severity of the volume changes and is probably related to the remarkable degree of recovery shown by the cells

The origin of the increase in volume of the interstitial fluid is most likely to be osmotic in nature This is not to say that there is normally a high electrolyte concentration in the extracellular fluid which attracts water when strial transport is deranged because this does not seem possible in view of the facilitated exchange between the fluid and the capillaries as revealed by tracer studies (Duvall et al 1971 Osako & Hilding 1971 Winther 1971) For the same reason the production of severe pressure changes causing forceful separation of the cells is impossible to envisage What probably happens is a redistribution of electrolyte and secondarily to preserve isotonicity water between the marginal and intermediate cells on the one hand and the interstitial fluid on the other this being due to the inhibition of the various membrane bound transport processes much as suggested by Brummett et al (1977)

During the development of the intoxication the shift is from the intermediate cells to the spaces and the marginal cells no spaces being observed where the intermediate cells were normal On recovery the reverse takes place Here the marginal cell compensation was significantly more rapid than the intermediate cell re-enlargement This led temporarily to a secondary increase in the size of the interstitial spaces an increase which had no equivalent in the physiological effects (Bosher 1980 and Fig 1) The volume of the strial extracellular fluid therefore almost certainly depends on a number of complex factors operating largely independently in the two types of cell The interstitial space variations consequently do not provide a reliable indication of any single specific action of the drug

The scattered translucencies in the capillary basement membrane confined to the initial stages have also been described in the guinea pig (Brummett et al 1977) and may represent a direct effect of the drug which passes off as the blood level falls Anecdotal accounts (Duvall et al 1974 Kasajima 1973) suggest anomalies in the passage of horse radish peroxidase might occur at this time but the details are contradictory and the matter needs exploring further The shortness of the effect s duration would seem to exclude it from having a major role if it has one at all

CONCLUSIONS

When the three principal functional effects of ethacrynic acid (Bosher 1980) are considered the present investigation has revealed that there is a good correlation in the rat between the onset and recovery of the inhibition of the strial transport processes on the one hand and the development and regression of the marginal cell swelling on the other The findings in other tissues confirm this swelling to be a useful index of the progress of the ethacrynic acid intoxication

The scattered mitochondrial changes seem related to the probable delayed decrease in energy production While such a conclusion is supported by the more extensive structural alterations described in the guinea pig their low incidence in the rat is not sufficient alone to establish the correlation although it is consistent with it Normal looking mitochondria

ts clearly do not exclude functional derangements.

The decrease in the overall cation permeability of the endolymph system which persists without any evidence of recovery for at least 120 min has no morphological correlate in the stria. In general one would not be expected but it is also not certain whether the permeability perturbations arise elsewhere in the cochlear duct. Conversely the rapid involvement and slow progressive recovery of the intermediate cells does not have a functional counterpart. The drug's action upon these cells once more stresses our lack of knowledge about them.

The effects of ethacrynic acid are clearly of some complexity, an aspect further emphasized by the late structural abnormalities which arise in the stria after apparent functional recovery (Brummett et al. 1977) and by the report of persistent changes for extremely long periods in some circumstances (Silverstein & Begin 1974). It is this complexity which detracts from the usefulness of ethacrynic acid as a tool for delineating the normal endolymph-controlling mechanisms. In this respect, therefore, ouabain might be of greater value in view of its more restricted mode of action. Surprisingly the structural alterations produced by ouabain do not seem to have been studied in mammals and so such an investigation is now in progress in this laboratory.

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33 Ratten erhielten eine einmalige intravenöse Injektion von Ethacrynsäure (60 mg kg⁻¹). Zu verschiedenen Zeiten bis zu 120 Minuten nach der Injektion wurde die Beziehung der ultrastrukturellen Veranderungen in der Stria vascularis zu den funktionellen Veranderungen im Endolymphsystem untersucht. Der Gesamteffekt auf die marginalen Zellen (erscheinend im Auftreten und Zurückgehen ausgesprochener Zytoplasmaschwellung) stand recht gut in Beziehung zu den Störungen im Ionentransportmechanismus der Striae. Die patho-physiologischen Beziehungen der Wirkung in den intermediären Zellen war nicht so klar und strukturelle Korrelationen zur Veranderung der membranösen Permeabilität wurden nicht gefunden, auch nicht zu der wahrscheinlichen, späteren Veranderung der produzierten Energie. Die Große der interstitiellen Zwischenräume wurde in der Hauptsache auf Grund der Variationen im Volumen der marginalen und der intermediären Zellen geschätzt.

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S A Boshe
Ferens Institute of Otolaryngology
The Middlesex Hospital Annexe
Cleveland Street
London WIP 5FD
England

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INHIBITION OF Na K STIMULATED ATPase IN THE COCHLEA OF THE GUINEA PIG

A Potential Cause of Disturbed Inner Ear Function in Terminal Renal Failure

D Adler W Fiehn and E Ritz

From the ENT Clinic and the Department of Internal Medicine University of Heidelberg FRG

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Abstract The frequent occurrence of sensorineural hearing loss in patients with chronic renal insufficiency prompted us to study the influence of chronic renal failure upon N ,K ATPase in the inner ear of guinea pigs. N ,K -activated ATPase was defined as the ouabain-sensitive part of total ATPase the activity of which was obtained in the presence of sodium, potassium and magnesium A significant reduction of N ,K -activated ATPase was found in the inner ear of uremic animals Such inhibition could be demonstrated as early as 1. hours after subtotal nephrectomy An inverse correlation was found between serum creatinine levels and Na ,K activated ATPase. A similar inhibition of N ,K -activated ATPase in uremia is also found in other tissues (erythrocytes, renal tubules, intestinal mucosal cells, sarcolemma) N ,K ATPase in the cochlea plays a key role in the maintenance of cochlear cationic gradients. It is suggested that inhibition of this enzyme system may contribute to the inner ear dysfunction in uremia.

Audiometric studies frequently show disturbed inner ear function in patients with terminal renal failure (Yassin et al 1966 Kopsa et al 1972 Bergstrom et al 1973 Mitschke et al 1975 Quick 1976) The cause of such lowered auditory acuity has not been established The high sensivity of cochlear hair cells to mechanical stimulation seems to depend on the ionic composition of the endolymph (Konishi et al 1966) Maintenance of the characteristic cation concentration gradients between endolymph and perilymph (Smith et al 1954) and the positive endolymphatic potential (Békésy 1960) are related to the activity of Na K -stimulated ATPase as reported by Kuijpers (1969) This corresponds to what is found in many other cell systems Na K -stimulated ATPase of the cochlea is susceptible to inhibition by ouabain (Kuijpers 1969)

In patients with terminal renal failure and in animals with experimental uremia, changes in the activity of Na K stimulated ATPase were demonstrated in various organ systems. Such inhibition of Na ,K ATPase activity was found in erythrocytes (Welt et al 1964 Tanaka, 1967 Villamil et al 1968 Cole 1973) and in the plasma membrane of heart muscle (Fiehn et al 1976) Furthermore increased ATP concentrations were found in intestinal mucosa (Kramer et al 1976) and this was thought to be due to inhibition of Na K stimulated ATPase Bricker et al (1970) concluded that the activity of this enzyme in chronic renal failure is due to the presence of a circulating inhibitor in the blood Such inhibition may cause natriuresis in the kidney and may contribute to sodium homeostasis in individuals with compromised renal function

It is conceivable that such hypothetical natriuretic substance in individuals with terminal renal failure may also exert an inhibitory effect on Na ,K -stimulated transport ATPase in the inner ear The present investigation was undertaken to examine the question whether the activity of transport ATPase of the cochlea is affected in experimental uremia.

Table I *Protocol of the experiments showing serum urea and creatinine levels ATPase activities and the stimulation of the ATPase by alkali ions in %*

The specific Na K -stimulated part is defined as the ouabain sensitive part of total ATPase activity For experimental details see Methods and Materials

No		Time post op	Serum urea (mmol/l)	Serum creat. (µmol/l)	ATPase Basal	Stim.	Total	% stimulation of basal ATPase by Na and K
					(µmol P×mg prot.×hour)			
1	Control	1[illegible] h	7 8[illegible]	–	1 08	0.25	1.33	23 15
	5/6 N	1 h	18.31	–	0 96	0 18	1 14	18 75
3	5/6 N	1 h	17.32	–	0.96	0 12	1 08	1[illegible]5
4	5/6 N	12 h	21.31	–	1 02	0 12	1 14	11 76
5	5/6 N	12 h	19 48	–	1 79	0.24	2 03	13 40
6	Control	4 h	7 49	53 04				37.5
7 r	5/6 N	24 h	23 48	141 44				6.0
8 l	5/6 N	24 h	23 48	141 44				8.36
9	Control	24 h	4 66	–	0 7[illegible]	0 12	0 84	16 66
10	Control	4 h	5 16	–	1 68	0 48	2 16	28.57
11	5/6 N	24 h	26 81	–	0.90	0 1	1 02	13.33
1	5/6 N	4 h	32 63	–	1.80	0.24	04	13.33
13	Control	2 d	7.33	–	1.26	0.30	1.56	[illegible]3 81
14	5/6 N	d	34 46	–	0 78	0 16	0.96	20 51
15	5/6 N	d	35.30	–	0 90	0 1	1 02	13.33
16	Control	4 d	7 49	53 04	0 672	0 144	0.816	21 4[illegible]
17	Control	4 d			0 639	0.213	0 852	33.33
18	Control	4 d	8.82	53 04	0 485	0 130	0 615	26.80
19	Control	4 d			0 449	0 142	0.591	31 63
20	Control	4 d	9 49	44	0.280	0 059	0.339	21.07
1	Control	4 d			0.304	0 070	0.374	23 03
22	Control	4 d	9 49	53 04	0 22[illegible]	0 047	0.269	21 17
[illegible]3	5/6 N	4 d	33 3	159 1	0 14	0 011	0 153	7 75
4	5/6 N	4 d			0 15[illegible]	0 006	0 158	3.95
25	5/6 N	4 d	33 3	150.28	0 690	0.035	0 725	5.07
	5/6 N	4 d			0 315	0 024	0.339	7 6[illegible]
26	Control	7 d	7 16	35 6	0.245	0 089	0.334	36.33
27	5/6 N	7 d	14 49	70 7[illegible]	0[illegible]89	0 039	0.328	13 49
28	5/6 N	7 d	10.3[illegible]	70 72	0 144	0 023	0 167	15 97
[illegible]9	5/6 N	7 d	15 48	53 04	0.228	0 049	0.27[illegible]	19.30
30	Control	15 d	7.99	35 6	0.301	0 098	0.399	32.56
31	5/6 N	15 d	17.32	70 72	0.322	0 063	0.385	19.57
32	5/6 N	15 d	33.3	97 24	0.240	0 038	0 [illegible]78	15.83
33	5/6 N	15 d	11 66	79.56	0[illegible]51	0.045	0.296	17.93
34	Control	22 d	8 16	44 2	0[illegible]47	0 070	0.317	[illegible]8.34
35	Control	22 d	8.99	53 04	0.207	0 055	0.262	6.57
36	5/6 N	22 d	15 65	88 4	0 294	0 035	0.3[illegible]9	11.9
37	5/6 N	22 d	15.32	79.56	0 235	0 023	0.258	9 79
38	5/6 N	22 d	14 49	79.56	0.388	0.023	0 411	5.93
39	5/6 N	22 d	23[illegible]4	150 28	0 163	0 02[illegible]	0 185	13.50

METHODS AND MATERIALS

280–400 g male guinea pigs with a normal Preyer reflex were used throughout the experiments 16 animals were subjected to sham operation (decapsulation of the kidney) and served as controls The other animals were subjected to subtotal (5/6) resection of renal parenchyma under Nembutal® anaesthesia (Pentobarbital-Natrium 31 mg/kg) Both kidneys were exposed from a 4 cm dorsal incision The upper and lower poles of the left kidney were resected and coagulated care being taken that hilus and adrenal glands were

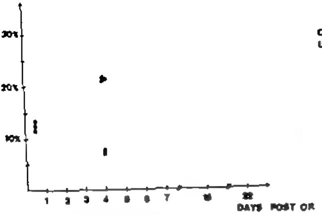

Fig. 1. ATPase stimulation by sodium and potassium (%) of the cochlea from control (closed symbols) and uremic guinea pigs (open symbols) various times after sham operation and subtotal nephrectomy.

not damaged. The right kidney was removed leaving the adrenal glands *in situ*. All animals were given pig chow and deionised water *ad libitum*.

At the end of the experiment, blood was taken from the abdominal aorta for determination of urea and creatinine on a Technicon Auto Analyser. Immediately after decapitation the bullae were removed. Under the operating microscope the bullae were opened and the bony otic capsule was removed by dissection. The membranous cochlear structures of the three turns were homogenized in 1 ml aqua bidest. at 0°C. Contamination of the cochlear homogenate by small osseous fragments containing bone ATPase was avoided by careful preparation under the dissecting microscope. Microscopical examination of the cochlear structures failed to reveal any bone fragments. The time required for preparation was identical in uremic animals and controls.

In the homogenate protein concentration was determined according to Lowry (1951). ATPase activity was determined at 37°C after 20 minutes of incubation. The reaction was stopped by the addition of TCA and inorganic phosphate, cleaved from ATP, was measured as described by Fiske & Subba Row (1925). For total ATPase activity incubation medium contained 60 mM NaCl, 6 mM KCl, 30 mM Tris-HCl pH 7, 3 mM $MgCl_2$ and 3 mM Tris-ATP (incubation volume 1 ml). Basal or Mg^{++} dependent ATPase was measured in the above medium with 0.1 mM ouabain additionally present. In preliminary experiments it could be demonstrated that by this concentration of ouabain the Na ,K -stimulated fraction of ATPase is completely inhibited. Specific Na ,K -stimulated ATPase was calculated from the difference between total ATPase and basal ATPase.

RESULTS

With the technique used, serum creatinine levels 4 days after subtotal nephrectomy were three times and serum urea levels four times higher in uremic as compared with sham-operated control animals. Subsequently creatinine and urea levels decreased, presumably due to compensatory growth of the remaining parenchyma. After 22 days the elevation of serum urea and of serum creatinine was rather modest.

The results showing the effect of subtotal nephrectomy are shown in detail in Tables I and II. The activity of the basic ATPase, obtained in the presence of Mg^{++} only, was identical in the preparation from uremic and sham-operated animals. In contrast, a marked inhibition of the sodium- and potassium-dependent stimulation of this enzymatic activity was found as early as 12 to 24 hours after subtotal nephrectomy, being demonstrable even 22 days after the resection (Fig. 1). The mean stimulation of the ATPase by alkali ions in all

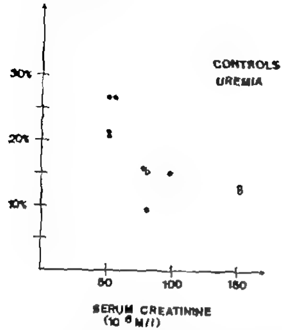

Fig 2 Relation between serum creatinine levels and Na ,K -dependent ATPase stimulation (%) of the cochlea of control guinea pigs (closed symbols) and guinea pigs with experimental uremia (open symbols)

the uremic animals (irrespective of the duration of uremia) was with 13 2% (range 6 1–17 8%) significantly reduced when compared with 27 4% (range 23 2–36 3%) in control animals There was an inverse relation between serum creatinine levels and Na^+,K^+ stimulation (Fig 2) whereas no such a clear-cut correlation could be demonstrated for serum urea levels

DISCUSSION

The above results clearly demonstrate inhibition of Na^+ K^+-stimulated ATPase in the cochlea of guinea pigs with experimental renal failure The result is the more remarkable since the degree of renal failure achieved with the above surgical technique was rather modest As control animals were only subjected to sham operation a non specific effect of stress appears unlikely Furthermore the results cannot be explained as the non-specific effect of fasting since for up to 12 hours postoperatively animals refused to take any food whereas even at this time a marked depression of enzymatic activity was already demonstrable in uremic animals

Artefacts resulting from the operation of the cochlea appear unlikely Great care was taken to avoid contamination of the membranous cochlear structures by bone fragments In addition the duration of preparation was kept identical in uremic and sham-operated control animals Since Na^+ K^+ ATPase is relatively stable (Kuijpers) a marked loss of enzyme activity during the short duration of preparation which did not exceed 15 min is unlikely

The inhibition of Na^+ K stimulated ATPase in our experiments should cause an increase of sodium and a decrease of potassium concentration in the endolymph with a concomitant change of transmembrane potential As a result fluid transport in the inner ear should also be disturbed Merck et al (1976) found morphological evidence for disturbed ion and fluid transport in the inner ear of uremic rats They observed marked swelling of intermediary cells Particularly striking was an increased size of the mitochondria of intermediary cells and endothelial cells of the stria vascularis

The relation between Na^+ K^+ stimulated ATPase and transmembrane Na and K transport or endolymphatic potential has not yet been completely elucidated In the cochlea of guinea pigs Iinuma (1967) and Kuijpers (1969) found that the highest activity of Na^+ K^+ stimulated ATPase was localized in the stria vascularis Kuijpers (1969) showed that ouabain simultaneously decreases Na^+ K -stimulated ATPase endolymphatic potential and cochlear microphone potentials Thalmann et al (1977) as well as Horn et al (1978) found that after ethacrynic acid application endolymphatic potentials and activity of Na K^+-stimulated ATPase decreased However the threshold concentrations of ethacrynic acid which caused the decrease in potentials or inhibition of enzyme activity were strikingly different (Thalmann et al 1977) Therefore the exact relati between

the transport of sodium and potassium and Na–K stimulated ATPase in the cochlea remains to be established.

The observed inhibition of Na–K-stimulated ATPase in the cochlea of uremic animals parallels findings obtained with other organs, e.g. erythrocytes (Welt et al., 1964; Tanaka, 1967; Villamil et al., 1968; Cole, 1973), intestinal mucosa (Kramer et al., 1976) and sarcolemma of the myocardium (Fiehn et al., 1976), where inhibition of Na–K-stimulated ATPase was also demonstrated. The cause of such inhibition has not been defined. Welt et al. could show that the disturbance of translocation of sodium across the membrane of erythrocytes could be abolished by one single hemodialysis. This finding suggests that accumulation of a water-soluble, low molecular weight dialysable substance is at least in part responsible for the inhibition of the enzyme.

The nature and possible function of such an inhibitory factor was elucidated by Grantham et al. (1973). These authors studied the effect of the serum of uremic patients on fluid transport in isolated proximal tubuli of the kidney and found that it resulted in marked inhibition of Na and fluid reabsorption. Flanigan et al. (1978) studied the action of serum of uremic patients on sodium transport in the frog skin and also found a depression of sodium transport. Both these observations suggest the presence of a circulating natriuretic factor in the serum of uremic patients. Such a natriuretic factor has been postulated by Bricker et al. (1970) and was thought to be important for sodium homeostasis in the uremic organism. It remains to be shown whether such natriuretic substance is identical with the hypothetical inhibitor of Na–K stimulated ATPase in the inner ear.

ZUSAMMENFASSUNG

Bei Patienten mit terminaler Niereninsuffizienz wird häufig eine Innenohrschwerhörigkeit beobachtet. Wir prüften experimentell die Hypothese, daß der Störung bei terminaler Niereninsuffizienz eine Hemmung der Transport-ATPase im Innenohr zugrunde liegt. Die Na–K-stimulierte ATPase in der Meerschweinchen-Cochlea wurde quantitativ bestimmt. Wie in anderen Geweben (Erythrozyten, Sarkolemm) fand sich bei terminaler Niereninsuffizienz in der Cochlea bei unveränderter basaler ATPase eine Hemmung der Na–K-stimulierten Transport-ATPase. Die Enzymhemmung war bereits 12 Stunden nach 5/6-Nephrektomie nachweisbar; die mittlere Enzymaktivität bei den niereninsuffizienten Meerschweinchen war etwa um die Hälfte vermindert. Es bestand eine inverse Beziehung zwischen Kreatininspiegel und Na–K-ATPase-Aktivität. Eine Hemmung des Na–K-ATPase-Enzymsystems, das eine Schlüsselrolle bei der Aufrechterhaltung der cochleären Ionen-Gradienten einnimmt, könnte die Störung der Innenohrfunktion bei Niereninsuffizienz erklären.

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D D Adler
Universitats-HNO-Klinik
69 Heidelberg
Voss Str 5–7
Bundesrepublik Deutschland

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POTENTIATION OF NOISE INDUCED AUDIOGENIC SEIZURE RISK BY SALICYLATE IN MICE AS A FUNCTION OF SALICYLATE–NOISE EXPOSURE INTERVAL

Chia-Shong Chen and G Clare Aberdeen

From the Department of Psychology Monash University Clayton, Victoria, Australia 3168

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Abstract Audiogenic seizure risk can be induced in genetically seizure-resistant BALB/c mice by exposure to an intense noise Results of this experiment showed that combined exposure to noise and sodium salicylate could produce a greater priming effect than exposure to the noise alone and the greatest potentiation effect was obtained when animals were exposed to the noise 6 hr after the peak of salicylate The findings were taken as indirect evidence suggesting that the ototoxic action of sodium salicylate could potentiate vulnerability of the mouse cochlea to noise damage

Whether simultaneous exposure to noise and ototoxic drugs can result in a mutual potentiation of their oto-traumatic effect is an important practical as well as theoretical issue Available evidence suggests that this potentiation can occur if irreversible ototoxic drugs such as kanamycin or neomycin are involved (Hamernik and Henderson 1976) It is less clear whether similar effects can be obtained when reversible ototoxic drugs such as salicylate are used. General consensus is that combined exposure to salicylate and noise should not pose an additional hazard to the auditory system (Hamernik & Henderson 1976) However a recent experiment (Eddy Morgan & Carney 1976) indicated that combined exposure to salicylate and noise could cause a greater temporary hearing loss (55 dB) than exposure to either agent alone (35 dB and 30 dB respectively for noise and salicylate) Research concerning a longer term effect of combined exposure to noise and salicylate has not been reported The general aim of this study was to investigate this problem by using a new animal model namely the induction of audiogenic seizure risk by an intense noise in genetically seizure resistant BALB/c mice (Chen 1973) The rationale for using this phenomenon for evaluation of the parameters of noise effect is discussed below

An intense noise can cause not only a temporary loss in the hearing sensitivity of BALB/c mice but also a drastic change in the reactivity of their auditory systems to an intense noise (Chen 1978) This drastic change is reflected at the neurophysiological level by a decrease in sensitivity to soft sounds (e g. reduction in the size of evoked potential in the cochlear nucleus) and a drastic increase in sensitivity to intense sounds (e g. close to two-fold increase in the magnitude of evoked potentials) as compared with the control animals (Saunders, Bock James & Chen 1972) At the behavioral level it is reflected by an increased propensity to show audiogenic seizures when the noise-exposed animal is re-exposed to an intense stimulus (Chen 1973) or by an augmentation of the magnitude of the acoustic startle response (Chen 1978) An initial exposure to the noise is absolutely necessary for this drastic change to occur as sham-exposed littermates showed none of these behavioral and physiological reactions.

An audiogenic seizure is a sequence of abnormal psychomotor responses elicited by an intense acoustic stimulus. It consists of wild running followed by a clonic seizure which may then developed into a more severe state

of tonic seizure (Fuller & Collins 1970) It is known that certain strains of inbred mice are either genetically susceptible (e g DBH/2 mice) or un susceptible (e g BALB/c mice) to audiogenic seizures (Fuller & Collins 1970) However susceptibility to audiogenic seizures can be reliably induced in many genetically non-susceptible strains of mice (e g C57BL/6J mice—Henry 1967 BALB/c mice—Chen 1973) by exposure to a loud noise several days prior to testing for seizures This phenomenon has been termed priming or sensitization for audiogenic seizure risk

Evidence exists which suggests that priming for audiogenic seizure risk could be a useful and valid animal model for the investigation of noise induced stimulation damage to the cochlea 1 Histological evidence indicates that an effective priming exposure causes ex tensive damage to the cochlear outer hair cells particularly of the basal turn (Norris Cawthon & Carroll 1977) 2 Primed mice show a severe loss in cochlear sensitivity as in dicated by their poor cochlear microphonic response (Saunders Bock Chen & Gates 1972) 3 Audiogenic seizure risk can be in duced by ototoxic drugs such as kanamycin (Norris et al 1977) or 6-Aminonicotinamide (Chen and Gates 1977) 4 The parameters of the priming stimulus are found to be positively correlated with parameters of stimulation damage to the cochlea For example an in crease in intensity (Chen 1973) in duration of priming (Gates & Chen 1975) or in acoustic energy (Graham 1977) tends to increase the effectiveness of priming All these experi mental findings indicate that induced audio genic seizure risk is closely related to patho logical conditions of the mouse cochlea. Thus it appears reasonable to suggest that priming for audiogenic seizures is a valid indirect method for evaluations of the ototraumatic ef fect of noise

It was found recently in this laboratory that combined exposure to noise and salicylates (either sodium salicylate or acetyl salicylic acid) could produce a greater priming effect than exposure to either ototoxic agent alone. There are three possible ways for this en hanced priming effect to occur 1 It could be the result of a simple independent summation of their separate ototoxic effects. 2. The oto toxic action of salicylate could potentiate the vulnerability of the cochlea to noise damage 3 Salicylate could exert its detrimental effect after the noise exposure e g hindering re covery process and/or worsening the degen eration process This study was designed to evaluate these possibilities

METHOD

134 BALB/c mice 23 days of age were primed for audiogenic seizure by exposure to a 125–127 dB (re 0 0002 dyne/cm²) bell sound for a duration of 10 sec Seven days after priming, all mice were tested for audiogenic seizures by re-exposure to the same bell sound for 60 sec or until seizures occurred Types of seizure responses (wild running clonic and tonic seizures and death) as well as latencies to wild running and clonic seizure were recorded De tailed procedure for priming and testing for audiogenic seizure has been reported else where (Chen 1973)

Each mouse was injected orally with 500 mg/kg of sodium salicylate either 24 hrs or 6 hrs prior to the priming exposure (salicylate-noise groups) or either 1 hr or 24 hrs after the priming exposure (noise–salicylate groups) Two noise exposure alone groups were also run The noise alone animals were injected with water either 6 hrs prior to or 1 hr after the priming exposure Since these two groups showed no differential effect they were com bined to form a single noise alone group Each mouse was assigned to one of the above condi tions on a split litter basis

In order to improve the sensitivity of de tecting a potentiation effect it is essential that the acoustic priming stimulus should not be highly effective Otherwise the results would be confounded by the problem of a ceiling ef fect Results of a pilot study indicated that the

Table I *Induction of audiogenic seizure risk as a function of the interval between salicylate intake and priming exposure*

Condition	N	Incidence of seizure response WR	CL	CT
Noise alone	27	3	1	
Salicylate administered				
24 hrs before noise	27	7	5	3
6 hrs before noise	27	16	8	5
1 hr after noise	27	5	2	
24 hrs after noise	26	3	1	

parameters of the priming stimulus used had met this requirement

RESULTS AND DISCUSSION

Table I summarizes the incidence of audiogenic seizure responses for each of the experimental conditions. The result indicates that the noise exposure alone was not sufficiently severe to induce a high level of seizure risk Only 11% of the primed mice showed wild running and one mouse showed clonic seizure Similar results were obtained when sodium salicylate was injected either one hr or 24 hrs after the priming exposure A slight increase in the proportion of animals showing wild running (26%) clonic (19%) and tonic (11%) responses occurred when sodium salicylate was injected 24 hrs prior to the noise exposure The greatest priming effect was obtained when animals were exposed to the intense noise 6 hrs after administration of sodium salicylate A χ-square test indicated a significant overall group difference in the proportion of animals showing wild running ($\chi^2=22.57$ df$=4$ $p<0.001$) The number of subjects showing clonic seizures was too small to permit use of the χ^2 test However applications of the Fisher exact probability test indicated that the proportion of animals showing clonic seizures in the 6-hr salicylate group was significantly different from that of the noise alone or the 24-hr post-noise exposure groups ($p<0.02$, two-tailed) It also differed from the 1 hr post noise exposure group at 0 064 level of significance (two-tailed test)

Thus the result of the present study confirms a previous finding that combined exposure to salicylate and noise can produce a greater priming effect than exposure to the noise alone Since effective priming is known to depend upon the degree of cochlear dysfunction (reviewed in Introduction) the present finding may be taken as evidence suggesting that the salicylate intake could potentiate the ototraumatic actions of the noise Norris et al (1977) reported that noise that induced hair cell damage of 30–40% was not sufficient for seizure risk to develop only when the damage reaches the level of 70–90% of the basal turn outer hair cells could the seizure reactions be obtained It is of interest to note that a priming effect comparable to that induced by the 6-hr salicylate–noise treatment can be obtained by a 20% increase in the duration of exposure to the noise (i e 1? sec)

The fact that testing for seizures was conducted 7 days after exposure to the salicylate and noise suggests that the salicylate enhanced priming effect was relatively permanent. It also suggests that the enhanced effect was not caused by possible neurotoxic actions of sodium salicylate because by the time of testing the drug should have been fully metabolized and excreted. In addition, if the enhanced seizure risk was due to the neurotoxic action of the salicylate then one would expect the 4-hr noise–salicylate group which had an intervening interval of 6 days to show a greater priming effect. The result indicates that this is not the case

Salicylate and noise could independently produce two separate ototoxic effects which summate to produce the enhanced priming effect Alternatively these two ototraumatic agents might interact in a certain way to produce a greater cochlear dysfunction and hence greater priming effect If a simple summative action was involved one would expect that the temporal order of exposure to these two

of tonic seizure (Fuller & Collins 1970) It is known that certain strains of inbred mice are either genetically susceptible (e g DBH/2 mice) or un susceptible (e g BALB/c mice) to audiogenic seizures (Fuller & Collins 1970) However susceptibility to audiogenic seizures can be reliably induced in many genetically non susceptible strains of mice (e g C57BL/6J mice—Henry 1967 BALB/c mice—Chen 1973) by exposure to a loud noise several days prior to testing for seizures This phenomenon has been termed priming or sensitization for audiogenic seizure risk

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view, the present finding indicates that salicylate could pose additional risk for organisms exposed to an intense noise.

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ZUSAMMENFASSUNG

Werden genetisch anfallswiderständige BALB/c-Mäuse intensivem Lärm ausgesetzt, so erhöht sich das Risiko eines audiogenen Anfalles. Die Ergebnisse dieses Experimentes zeigten, daß ein größerer „Priming"-Effekt erreicht wird, wenn die Mäuse einer Kombination von Lärm und Sodium-Salicylate ausgesetzt wurden, verglichen mit Lärm allein. Der größte Effekt zeigte sich, wenn die Tiere dem Lärm 6 Stunden nach der Eingabe von Salicylate ausgesetzt wurden. Dieser Befund wurde als indirekter Beweis angesehen, daß die ototoxische Wirkung von Sodium-Salicylate die Anfälligkeit der Gehörschnecke bei diesen Mäusen für Beschädigung durch Lärm erhöht.

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G. Clare Aberdeen
Dep. Psychology
Monash Univ.
Clayton, Victoria
Australia 3168

agents should not be a critical factor in affecting the outcome However the result indicates that a significantly enhanced effect was obtained only in mice injected with sodium salicylate 6 hrs prior to the noise exposure and not in mice treated under the other three combined exposure conditions

It is also of interest to note that by the time of testing the ototoxic effect of salicylate should have dissipated Experiments involving other animals have consistently showed that salicylate does not cause obvious structural lesions to the inner ear structures and its ototoxic effect usually disappears within 3 days of salicylate withdrawal For example administration of salicylate to patients with rheumatoid arthritis can cause a hearing loss of 20–40 dB for pure tones (250 Hz to 8 Hz) with complete recovery to normal sensitivity occurring 24 to 48 hours after salicylate withdrawal (Myers & Bernstein 1965) Similar effects were obtained in squirrel monkeys with recovery occurring 24 to 72 hours after intoxication Histological results indicated that no structural damage to the cochlea sensory cells nerve endings or the stria vascularis had occurred (Myers & Bernstein 1965) A significant hearing loss of 18 to 24 dB measured by an elevation of the threshold to pinna reflex has been found in salicylate intoxicated guinea pigs with auditory functions recovering within 3 days of salicylate wihtdrawal (Crifo 1975) Sodium salicylate has also been found to reduce neural evoked potentials recorded at the round window in cats (Silvestein Bernstein & Davis 1967) and in guinea pigs (Mitchell Brummett Hines & Vernon 1973) with recovery of the function again reported within 7 days (Mitchell et al 1973) Therefore the non-existence of salicylate action at the time of testing suggests that conditions for the development of the enhanced priming effect must be produced at the time or soon after the priming exposure

The fact that the potentiation effect was obtained only when the noise exposure occurred at a time (i e 6 hrs after injection) when the ototoxic effect of sodium salicylate had approached the asymptote (Mitchell et al 1973 Silvestein et al 1967) appears to favor an interactive interpretation Salicylates through their interferences with biochemical activity in the cochlea (Bernstein et al 1967) might have predisposed the cochlea to greater ototraumatic actions of the noise Experimentally it is difficult to determine conclusively if the interaction between the salicylate and noise occurs at the time of noise exposure or afterward because the action of salicylate could not be readily terminated soon after exposure to the noise If salicylate exerts its influence mainly after the priming exposure then one would expect the 1 hr noise–salicylate group to show the enhanced priming effect furthermore this effect should be greater for the 1 hr noise–salicylate group than for the 24-hr noise–salicylate group However this outcome was not found (see Table I) The fact that concurrent exposure to both agents (salicylate–noise condition) was more effective than dissociative exposure (noise–salicylate condition) seems to favor the predisposition speculation

It was argued in this correspondence that priming for audiogenic seizures in mice could be a valid and useful animal model for the investigation of the parameters of noise damage One advantage of using this model is that a large number of subjects can be run thus improving the sensitivity of detecting an experimental effect or statistically speaking reducing the probability of erroneously concluding that no treatment effect exists The result of this study demonstrates that noise and salicylate could combine to produce a greater priming effect than exposure to the noise alone The result also shows that the temporal interval between the salicylate intake and noise exposure is an important determinant of the potentiation effect This enhanced priming effect was assumed to be brought about by the action of salicylate on the mouse cochlea making it more vulnerable to the ototoxic action of the noise Contrary to the consensus

view the present finding indicates that salicylate could pose additional risk for organisms exposed to an intense noise

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agents should not be a critical factor in affecting the outcome However the result indicates that a significantly enhanced effect was obtained only in mice injected with sodium salicylate 6 hrs prior to the noise exposure and not in mice treated under the other three combined exposure conditions

It is also of interest to note that by the time of testing the ototoxic effect of salicylate should have dissipated Experiments involving other animals have consistently showed that salicylate does not cause obvious structural lesions to the inner ear structures and its ototoxic effect usually disappears within 3 days of salicylate withdrawal For example administration of salicylate to patients with rheumatoid arthritis can cause a hearing loss of 20–40 dB for pure tones (250 Hz to 8 Hz) with complete recovery to normal sensitivity occurring 24 to 48 hours after salicylate withdrawal (Myers & Bernstein 1965) Similar effects were obtained in squirrel monkeys with recovery occurring 24 to 72 hours after intoxication Histological results indicated that no structural damage to the cochlea sensory cells nerve endings or the stria vascularis had occurred (Myers & Bernstein 1965) A significant hearing loss of 18 to 24 dB measured by an elevation of the threshold to pinna reflex has been found in salicylate intoxicated guinea pigs with auditory functions recovering within 3 days of salicylate wihtdrawal (Crifo 1975) Sodium salicylate has also been found to reduce neural evoked potentials recorded at the round window in cats (Silvestein Bernstein & Davis 1967) and in guinea pigs (Mitchell Brummett Hines & Vernon 1973) with recovery of the function again reported within 7 days (Mitchell et al 1973) Therefore the non-existence of salicylate action at the time of testing suggests that conditions for the development of the enhanced priming effect must be produced at the time or soon after the priming exposure

The fact that the potentiation effect was obtained only when the noise exposure occurred at a time (i e 6 hrs after injection) when the ototoxic effect of sodium salicylate had approached the asymptote (Mitchell et al 1973 Silvestein et al 1967) appears to favor an interactive interpretation Salicylates through their interferences with biochemical activity in the cochlea (Bernstein et al 1967) might have predisposed the cochlea to greater ototraumatic actions of the noise Experimentally it is difficult to determine conclusively if the interaction between the salicylate and noise occurs at the time of noise exposure or afterward because the action of salicylate could not be readily terminated soon after exposure to the noise If salicylate exerts its influence mainly after the priming exposure then one would expect the 1 hr noise–salicylate group to show the enhanced priming effect furthermore this effect should be greater for the 1 hr noise–salicylate group than for the 24-hr noise–salicylate group However this outcome was not found (see Table I) The fact that concurrent exposure to both agents (salicylate–noise condition) was more effective than dissociative exposure (noise–salicylate condition) seems to favor the predisposition speculation

It was argued in this correspondence that priming for audiogenic seizures in mice could be a valid and useful animal model for the investigation of the parameters of noise damage One advantage of using this model is that a large number of subjects can be run thus improving the sensitivity of detecting an experimental effect or statistically speaking reducing the probability of erroneously concluding that no treatment effect exists The result of this study demonstrates that noise and salicylate could combine to produce a greater priming effect than exposure to the noise alone The result also shows that the temporal interval between the salicylate intake and noise exposure is an important determinant of the potentiation effect This enhanced priming effect was assumed to be brought about by the action of salicylate on the mouse cochlea making it more vulnerable to the ototoxic action of the noise Contrary to the consensus

view the present finding indicates that salicylate could pose additional risk for organisms exposed to an intense noise

ACKNOWLEDGEMENT

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ZUSAMMENFASSUNG

Werden genetisch anfallswiderständige BALB/ Mäuse intensivem Lärm ausgesetzt, so erhöht sich das Risiko eines audiogenen Anfalles. Die Ergebnisse dieses Experimentes zeigen, daß ein größerer „Priming" Effekt erzielt wird, wenn die Mäuse einer Kombination von Lärm und Sodium Salicylate ausgesetzt wurden, verglichen mit Lärm allein. Der größte Effekt zeigte sich, wenn die Tiere dem Lärm 6 Stunden nach der Eingabe von Salicylate ausgesetzt wurden. Dieser Befund wurde als indirekter Beweis angesehen, daß die ototoxische Wirkung von Sodium-Salicylate die Anfälligkeit der Gehörschnecke bei diesen Mäusen für Beschädigung durch Lärm erhöht.

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G. Clare Aberdeen
Dep. Psychology
Monash Univ.
Clayton, Victoria
Australia 3168

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ANALYSIS OF PROTEINS OF THE STRIA VASCULARIS OF THE NORMAL AND THE WALTZING GUINEA PIG

R J Wenthold and M L. McGarvey

From the Laboratory of Neuro-otolaryngology National Institute of Neurological and Communicative Disorders and Stroke National Institutes of Health Bethesda USA

(Received July 10 1979)

Abstract Proteins of the stria vascularis of the normal and the genetically deaf waltzing guinea pig were analysed by one and two-dimensional acrylamide gel electrophoresis Cochlear proteins were labeled in vivo by replacing the perilymph with a solution containing radioactive precursors. With the two-dimensional analysis, more than 200 polypeptides were resolved Proteins that are exposed on the endolymphatic surface of the stria vascularis were identified by lactoperoxidase-catalysed iodination Seven polypeptides were identified with this technique No consistent changes in protein patterns of the stria vascularis from the waltzing guinea pig were detected

There is little published information concerning the function and dysfunction of the peripheral auditory system at the molecular level The complexity and small size of cochlear structures have limited biochemical studies on this system Although its role in cochlear function has not been determined it is commonly thought that the stria vascularis is involved in both maintaining ionic conditions of the endolymph and generating the endocochlear potential (Sellick & Johnstone 1975 Thalmann 1975 Dallos 1975) The stria vascularis may be the initial site of action of some ototoxic drugs For example ultrastructural changes are apparent in the stria vascularis within minutes after administration of the ototoxic drug ethacrynic acid (Brummett et al 1977) In the dog a decrease in potassium and an increase in sodium in the endolymph is seen after administration of the drug (Cohn et al 1971) Furthermore some forms of genetic deafness have been linked to abnormalities of the stria vascularis (Schuknecht et al 1974) Studies of these disorders and other cochlear abnormalities may benefit from a characterization of cochlear proteins. Until now such studies have been largely limited to enzymes involved in metabolism (Thalmann et al 1970) or neurotransmitter synthesis (Fex & Wenthold 1976 Godfrey et al 1976)

The recently developed techniques of two-dimensional electrofocusing/electrophoresis (O Farrell 1975 Ames & Nikaido 1976) and fluorography (Bonner & Laskey 1974) allow separation and detection of several hundred proteins in samples containing only a few micrograms of protein In the present study we have investigated the proteins of the stria vascularis of the guinea pig and have identified those proteins on the endolymphatic surface of the stria vascularis by lactoperoxidase-catalysed iodination Proteins of the stria vascularis of the genetically deaf waltzing guinea pig were also studied

MATERIALS AND METHODS

The radioactive substances L-[4,5-^{3}H]-leucine (55 Ci/mmole) L-[5 6-^{3}H]-fucose (60 Ci/mmole) and L-[^{35}S]-methionine (500–600 Ci/mmole) were obtained from New England Nuclear Boston Mass ^{3}H leucine and ^{3}H fucose were lyophilized to dryness and ^{35}S-methionine was blown dry under N_2 at room temperature immediately before use Radioactive amino acids or fucose were resuspended in a solution c[illegible] 20 or 25 µl of 140 mM

NaCl 3.5 mM KCl 1 mM MgSO 3.3 mM glucose and 10 mM HEPES pH 7.4 NIH strain normal or waltzing guinea pigs 10–90 days of age were anesthetized with urethane (1 500 mg/kg) (and ether when necessary) and the round and oval windows were exposed Cochlear proteins were labeled by replacing the perilymph with the solution containing radioactive amino acids or fucose Window membranes were removed and a small amount of fluid was withdrawn through the oval window The solution containing the radioactive substance was added through the round window and the procedure repeated until the total volume of the solution 20 or 25 µl had been injected Bone wax was placed over the cochlea and animals were maintained on a heating pad Labeling times of 1 to 5 hours were used

The animals were killed by decapitation and the labeled cochlea was removed and placed in phosphate buffered saline (0.14 M NaCl 10 mM NaPO pH 7.4) PBS on ice Cochlear dissection was done in PBS with illumination from above The shell of the cochlea was chipped away with a No 11 surgical blade and the spiral ligament with the stria vascularis attached was removed usually in several pieces The stria vascularis was removed from the spiral ligament by holding the spiral ligament to the bottom of the Petri dish with forceps and peeling off the stria vascularis with a No 11 blade One cochlea was dissected in about 30 minutes. Although the solution was initially on ice the dissection was done at ambient temperature There is no indication this affected the protein patterns In two experiments in which the complete dissection was done on ice protein patterns identical with those done at room temperature were obtained

Endolymphatic surface proteins of the stria vascularis were labeled by carefully dissecting strips of spiral ligament with the stria vascularis attached from two cochleas The dissection was done in cold buffer 0.11 M K HPO (pH 7.4) and usually one complete strip was obtained from each cochlea The strips were transferred to 400 µl of 0.11 M K HPO (pH 7.4) and 25 µg of lactoperoxidase (100 IU/mg, Calbiochem La Jolla, CA) and 1 mCi of ^{125}I with 50 µM carrier NaI were added Ten µl of H_2O_2 (1.0 µM in 0.11 M K_2HPO_4) were added every 30 s for 10 min The reaction was then terminated by replacing the fluid with fluid containing 5 mM NaI The tissue was washed thoroughly and dissected in PBS as described above Control experiments in which the iodination was carried out in the absence of either lactoperoxidase or H_2O_2 showed no detectable labeling of strial proteins

One-dimensional gel electrophoresis was done using the method of Laemmli (1970) in either slabs (1.5 mm thick) or cylinders (6 mm diameter) Cylindrical gels were sliced frozen into 1 mm segments with a Bio-Rad gel slicer and solubilized with 30% H_2O_2. Two-dimensional electrofocusing/electrophoresis was done according to the method of Ames & Nakaido (1976) Samples were homogenized in 15 µl of a solution containing 2% SDS 0.5 mM $MgCl_2$ and 50 mM Tris HCl pH 7.2 and kept at room temperature 15 min. Thirty µl of a solution containing 9.5 M urea, 2% ampholines (0.4% pH 3.5–10 0.8% pH 4–6 0.8% pH 6–8) 5% mercaptoethanol and 8% NP-40 were added Five mg solid urea was then added to each sample Samples were electrofocused overnight at 400 V followed by 600 V for 1 h. Under these conditions a pH range of about 4.0 to 7.2 was obtained. The electrofocusing gels were then removed and frozen at −20°C or prepared immediately for electrophoresis as described by O'Farrell (1975) An equilibration time of 90 min was used Electrophoresis was carried out on a 10% acrylamide slab at 20 mA using the buffer system of Laemmli (1970) Gels were fixed stained and dried and proteins detected by fluorography (Bonner & Laskey 1974) Molecular weight standards used were γ-globulin, 160 000- phosphorylase b 94 000- bovine serum albumin 68 000 ovalbumin 45 000 chymotrypsinogen 25 000 D

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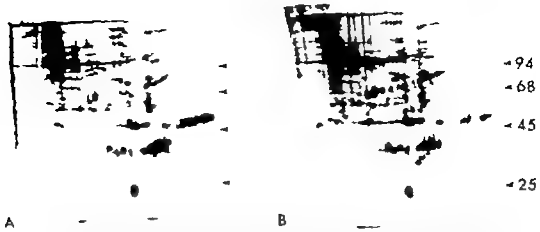

Fig. 3. Two-dimensional electrofocusing/electrophoresis of strial proteins of the normal (A) and waltzing (B) guinea pig. Animals were labeled with 150 μCi ^{35}S-methionine and killed 3 hours later. In the electrofocusing dimension, basic end of gel is to the left and acidic end to the right. Molecular weight standards are phosphorylase *b*, 94000; bovine serum albumin, 68000; ovalbumin, 45000 and chymotrypsinogen, 25000 D.

Two-dimensional analysis after fucose labeling shows groups of glycoproteins with each group consisting of several spots with similar molecular weights but differing in charge (Fig. 4B). For each group there is a slight increase in molecular weight as the isoelectric point decreases. These patterns are also detectable after ^{35}S-methionine labeling (Figs. 3, 6A). The charge and molecular weight heterogeneity is likely not due to interaction with ampholytes during electrofocusing, since one-dimensional analysis of fucose-labeled proteins produces broad peaks of radioactivity with molecular weights similar to those of the groups of spots seen with two-dimensional analysis. It is possible that these groups each represent glycoproteins with the same amino acid sequence but which vary in their amounts or compositions of sugar residues. A similar behavior has been reported for glycoproteins

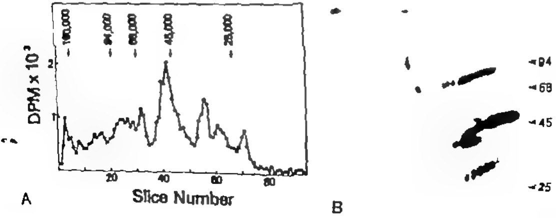

Fig. 4. One- (A) and two-dimensional (B) analysis of strial proteins 3 hours after injection of 200 μCi ^{3}H-fucose.

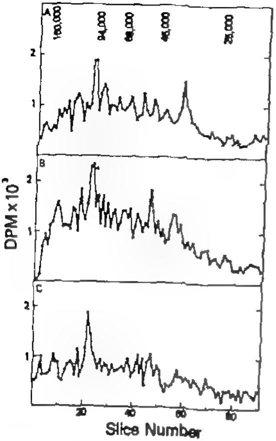

Fig 1 One-dimensional electrophoretic analysis of proteins of the stria vascularis at 1 (A) 3 (B) and 6 (C) hours after injection of 200 μCi ^{3}H-leucine

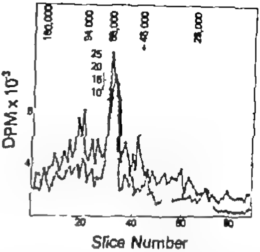

Fig 2 One-dimensional electrophoretic analysis of proteins of the spiral ligament 3 hours after labeling with either 200 μCi of ^{3}H-leucine (—), or 200 μCi ^{3}H-fucose (—)

RESULTS

Proteins of the stria vascularis were effectively labeled by replacing the perilymph with a solution containing radioactive amino acids or fucose ^{35}S-methionine and ^{3}H leucine are incorporated into growing peptide chains of proteins containing these amino acids while the incorporation of fucose is limited to glycoproteins Although leucine is generally more widespread in proteins the higher energy of ^{35}S makes methionine a better precursor when fluorography is used for analysis After injection of 150 μCi of ^{35}S-methionine the stria vascularis dissected from one cochlea containing about 4 μg protein incorporated about 300 000 dpm Differences in the degree of labeling could be at least partially attributed to leakage of radioactivity from the cochlea. Although the relative magnitudes of the peaks differ qualitatively similar electrophoretic profiles of strial proteins are seen at 1 3 and 6 hours after injection of ^{3}H leucine (Fig 1) Similar results were obtained when samples taken 1 3 and 6 hours after injection of ^{35}S-methionine were analysed by two-dimensional electrofocusing/electrophoresis (data not shown) ^{3}H leucine labeled proteins of the spiral ligament show a pattern markedly different from that of the stria. After injections of either ^{3}H-leucine or ^{3}H fucose the spiral ligament has a major peak of radioactivity migrating with a molecular weight of about 66 000 (Fig 2) The peak of fucose label consistently appeared at a slightly higher molecular weight than the leucine label suggesting that this peak may be composed of two or more distinct polypeptides

Two-dimensional electrofocusing/electrophoresis of proteins of the stria vascularis labeled with ^{35}S-methionine provides resolution of more than 200 polypeptides (Fig 3) The stria vascularis of normal and genetically deaf waltzing guinea pigs 10 to 90 days of age was studied with the two-dimensional method No consistent differences between gel patterns from the two animals were seen at any age investigated One-dimensional analysis of strial proteins labeled with ^{3}H fucose shows several broad peaks of radioactivity (Fig 4A)

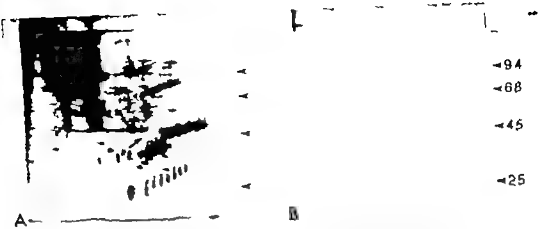

Fig. 6. Fluorograph of two-dimensional gel of ^{35}S-methionine labeled (A) or lactoperoxidase-catalysed iodinated (B) proteins of the stria vascularis. Arrows indicate ^{35}S-labeled polypeptides which co-migrate with the iodinated polypeptides. *Inset*: Higher magnification of 94 000 dalton iodinated polypeptide.

gion. This is illustrated by comparing Fig. 3 with Fig. 6A. In the present study 13 pairs of strial samples (10 labeled with ^{35}S-methionine and 3 with ^{3}H-fucose) were analysed by two-dimensional electrofocusing/electrophoresis and no consistent differences in protein patterns were seen between the normal and the genetically deaf waltzing guinea pig. However, it cannot be ruled out that there may be quantitative differences in individual proteins or that there are differences in minor proteins which are not detected with this technique. Ultrastructural analysis of the cochlea of the waltzing guinea pig shows that the earliest degeneration occurs in the organ of Corti, with no apparent strial involvement (Ernston 1971).

Lactoperoxidase-catalysed iodination of proteins allows identification of polypeptides located on the surface of membranes (Phillips & Morrison 1971, Hubbard & Cohn 1972). The reaction is catalysed by a macromolecule restricting labeling to the surface of the membrane provided the membrane is not damaged. Since the stria vascularis lies on the internal surface of the spiral ligament between the spiral prominence and Reissner's membrane, it can be removed from the cochlea undamaged attached to the spiral ligament. Previous studies have shown that the endolymphatic surface of the stria vascularis is not penetrated by proteins injected into the endolymph (Hinojosa, 1972). With this technique only a small number of polypeptides of the stria vascularis are labeled: 5 bands observed with one-dimensional and 4 with two-dimensional electrophoresis. The major proteins, as determined by ^{35}S-methionine incorporation (which correlate well with the Coomassie blue staining pattern) are not iodinated. The iodinated polypeptides co-migrate with relatively minor polypeptides as labeled with ^{35}S-methionine. Although glycoproteins are often located on the cell surface, none of the iodinated polypeptides co-migrate with strial proteins labeled with ^{3}H-fucose. The finding that most strial proteins are not iodinated indicates that only a specific class of polypeptides are accessible to the lactoperoxidase. Pretreatment of the stria vascularis with Triton X 100 (0.01% or 0.1% for 3 min) results in extensive labeling of additional polypeptides (unpublished observation). Since the iodination requires available tyrosine or histidine residues (Morrison & Bayse 1970; Phillips & Morrison 1971, Hubbard & Cohn 1972) it is possible additional polypeptides lacking these available amino acids are also located on the endolymphatic surface.

Since the stria vascularis is likely involved

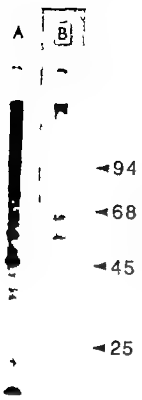

Fig 5 Fluorograph of strial proteins. A labeled with ^{35}S-methionine (150 μCi 3 h) and B labeled by lactoperoxidase catalysed iodination as described in Methods

from tissue culture cells (Baumann & Doyle 1979) With the fucose label no consistent differences were seen between gel patterns of strial proteins of the normal and the waltzing guinea pig (not shown)

To determine which if any of the polypeptides of the stria vascularis are exposed on the endolymphatic surface lactoperoxidase catalysed iodination of surface polypeptides was carried out on intact strial preparations Electrophoretic analyses of labeled proteins are shown in Figs 5 and 6 Five major bands corresponding to molecular weights of 103 000 95 000 65 000 55 000 and 45 000 are seen after one-dimensional electrophoresis (Fig 5) With the exception of the 103 000 band the labeled polypeptides appear to migrate closely with major strial proteins as labeled with ^{35}S-methionine Two-dimensional electrofocusing/electrophoresis shows four labeled spots migrating with molecular weights of 95 000 55 000 47 000 and 43 000 (Fig 6B) It is likely the 55 000 and 95 000 components are identical with those detected at the same molecular weights with the one-dimensional gel while the 45 000 component on the one-dimensional gel is resolved into two components of molecular weights 43 000 and 47 000 The 65 000 and 103 000 polypeptides detected in the one-dimensional analysis are not seen on the two-dimensional gel Perhaps they have basic isoelectric points and do not enter the electrofocusing gel By using the Coomassie blue staining pattern as a reference the iodinated polypeptides can be identified in the methionine labeled gels As seen in Fig. 6, those polypeptides that are iodinated appear as relatively minor species after ^{35}S-methionine label When the iodination was carried out in the absence of carrier NaI there was less radioactive incorporation but a similar pattern was obtained Also shorter labeling times gave similar gel patterns but correspondingly less incorporation Analysis of surface proteins of the stria vascularis of the waltzing guinea pig showed a pattern identical with that of the normal guinea pig (not shown)

DISCUSSION

The high resolution of two-dimensional electrofocusing/electrophoresis allows detection of more than two hundred radioactive polypeptides from a stria vascularis sample containing about 4 μg of protein Although qualitatively similar two-dimensional gel patterns were obtained between individual experiments the technique is not necessarily quantitative Several factors may account for variability including differences in *in vivo* labeling incomplete solubilization of proteins and loss of proteins during equilibration Variability was most frequently seen in the low molecular weight region likely due to protein loss during equilibration and in the high molecular weight acidic region likely due to incomplete solubilization of proteins in this re-

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Dr R J Wenthold
Laboratory of Neuro-otolaryngology NIH
Bldg 36 Rm 5D32
Bethesda MD 20205
USA

in maintenance of the ionic conditions of the endolymph proteins on its endolymphatic surface may be involved in this process The stria vascularis is a relatively rich source of two enzymes associated with ion transport carbonic anhydrase (Drescher 1977) and adenosine triphosphatase (ATPase) (Kuijpers et al 1967) Carbonic anhydrase is a soluble protein and not likely to be iodinated in the present experiments No strial polypeptides with molecular weights near that of carbonic anhydrase were iodinated ATPase a membrane-bound enzyme consists of two subunits with molecular weights of about 50 000 and 90 000–100 000 (Jorgensen 1974) Two iodinated polypeptides have molecular weights similar to those of the subunits of ATPase 55 000 and 95 000 Furthermore histochemical analysis of ATPase in the cochlea shows a reaction product on the endolymphatic surface of the stria vasclaris (Nakai & Hilding 1966) Present efforts are directed at further characterizing the endolymphatic surface proteins of the stria vascularis in normal and abnormal animals

The present results show that one and two-dimensional electrophoretic analysis of cochlear proteins after *in vivo* labeling may provide a very sensitive approach to the study of cochlear function This approach may be especially useful in elucidating the molecular mechanisms underlying such disorders as ototoxicity and genetic cochlear abnormalities Although in the present study about 200 spots were detected with two dimensional electrophoretic analysis additional minor polypeptides can be detected by expanding the electrofocusing gradient and developing the fluorographs longer Preliminary studies show that two-dimensional electrofocusing/electrophoresis can also be applied to the study of proteins of the organ of Corti

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ZUSAMMENFASSUNG

Proteine der Stria vascularis bei normalen sowie genetisch-tauben walzenden Meerschweinchen wurden mittels Akrylamidegel-Elektrophorese analysiert Kochleare Proteine wurden in vivo durch Ersetzung der Perilymphe durch eine Radioaktivvorgänger enthaltende Lösung markiert Mittels zweidimensionaler Analyse wurden über 200 Polypeptide zerlegt Proteine die auf der endolymphatischen Seite der Stria vascularis bloßgelegt werden, wurden mittels laktoperoxidasekatalysierter Jodverbindung nachgewiesen Mit dieser Methode wurden sieben Polypeptide identifiziert. Eine regelmäßige Änderung der Proteinmuster in der Stria vascularis walzender Meerschweinchen konnte nicht festgestellt werden.

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MATERIAL AND METHODS

Thirty two albino guinea pigs (200–300 grams) with active Preyer reflexes were studied Animals were anesthetized intraperitoneally with allobarbital-urethane (0 8 ml/kg body weight) and tracheotomized for artificial ventilation By a ventral surgical approach the left auditory bulla was opened and the cochlea was exposed. Glass micropipettes attached to polyethylene tubing were placed in the scala tympani and scala vestibuli of the basal cochlear turn to form a closed gravity-flow perilymphatic perfusion system (Nuttall et al. 1977) Approximately 50 μl of descending concentrations (0.5% – 0 0005 %) of HRP (Sigma Type VI) dissolved in artificial perilymph were perfused for between 5.5 hours and 15 seconds While the animal was still artificially ventilated the cochlear tissues were fixed *in situ* by perilymphatic perfusion of a freshly prepared 1.25% glutaraldehyde 1 0% paraformaldehyde solution (0.1 M sodium cacodylate buffered pH 7 4 4°C) *The right cochlea* was also exposed surgically and its tissues were similarly fixed

The temporal bones were dissected from the base of the skull and placed in fresh fixative for an additional 1.5 hours, whereupon they were washed in cold buffer (pH 7 4) over night Cochlear tissues were then microdissected from the bony labyrinth and collected in cold buffer The tissues were histochemically processed in a diaminobenzidine–hydrogen peroxide medium (Graham & Karnovsky 1966) A portion of the HRP-processed tissue was dehydrated and mounted in glycerol for light microscopical observations The remaining experimental tissues were post fixed in 1 0% osmium 0 1 M sodium cacodylate buffer (pH 7 4) for one hour and prepared for electron microscopy according to routine procedures Thick sections were cut from Epon-embedded tissues and examined by light microscopy Thick sections with HRP containing cells were re-embedded sectioned and collected on formvar-coated grids The sections were stained with uranyl acetate and lead citrate (Reynolds 1963) and examined in a Siemens Elmiskop 101 transmission electron microscope

RESULTS

The following experimental findings illustrate the effects of four different concentrations (0.5% 0 01% 0 005% and 0 0005%) of HRP and several postperfusion survival times (5.5 hr 1 hr 30 min and <30 min) on the albino melanocytes of the cochlear labyrinth They are representative of the effects of other tested concentrations of HRP and survival times.

A. *0.5% HRP*

Following a perilymphatic perfusion of a 0.5% concentration of HRP (postperfusion survival time = 5.5 hours) the cochlear tissues were heavily stained with HRP reaction product. The heaviest concentrations of the perfused exogenous peroxidase were located primarily in the spiral prominence and the tympanic crest portions of the spiral ligament In addition endogenous peroxidase was clearly identified in the red blood cells within the cochlear vessels in this and all other specimens examined

Numerous albino melanocytes were demonstrated along the blood vessels of the lateral cochlear wall particularly the suprastrial and the tympanic portions of the spiral ligament. Dendritic processes were observed to be in close proximity to adjacent blood vessels In some instances the dendritic processes of a single albino melanocyte contacted two adjacent vessels

The cell bodies of the albino melanocytes appeared darkly stained by the exogenous HRP and were markedly distended Their dendritic processes appeared club-shaped Many vacuoles and HRP bound vesicles were observed in the cytoplasm of the albino melanocytes (Fig. 1) Ultrastructurally *ruptured* limiting membranes and cellular vacuolization of the melanocytes were observed

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THE SENSITIVITY OF COCHLEAR ALBINO MELANOCYTES TO HORSERADISH PEROXIDASE

Kyle E Rarey and Muriel D Ross

F om the Department of Anatomy The U hersity of Michigan Ann Arbor Mich USA

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Abstract The effects of low concentrations (0.5%–0 0005%) of HRP and varying postperfusion survival times (5.5 hours – 15 seconds) on cochlear albino melanocytes were studied by light and electron microscopy Approximately 50 μl of differing concentrations of HRP dissolved in artificial perilymph were perfused through the perilymphatic space in 32 albino guinea pigs Tissues were subsequently treated histochemically to demonstrate HRP reaction product The results indicate that concentrations of HRP>0 0005% in combination with post perfusion survival times >1 hour bring about acute and selective injury to the albino melanocytes cytoplasmic vacuolization and rupture of cell membranes occurred The structural integrity of the albino melanocytes appeared unaltered when exposed to 0 0005% concentrations of HRP for ≤1 hour An apparently non-toxic 0 0005% concentration of HRP may provide a means of labeling cochlear melanocytes when the effects of other substances on the cells are investigated

Although non melanin-containing melanocytes (albino melanocytes Della Porta & Mühlbock 1966) have been demonstrated in the skin (Clark & Hibbs 1958) in hair follicles (Barnicot et al 1955 Barnicot & Birbeck 1958) and corneal epithelium (Baum 1970) of albino animals and humans the presence of albino melanocytes in the inner ear was until recently speculative (LaFerriere et al 1974) Ross et al (1977) reported the uptake of horseradish peroxidase (HRP) perfused through the perilymphatic space by cochlear albino melanocytes of the albino guinea pig However the experimental concentrations of HRP (ranging from 1% to 10%) caused varying degrees of cellular injury to the albino melanocytes other cochlear labyrinthine cells were less affected by the perfused HRP

The apparent sensitivity of cochlear albino melanocytes to HRP may help to augment the current knowledge of microhomeostasis in the inner ear Ross et al (1977) found a large population of albino melanocytes distributed in close proximity to the vasculature of the lateral cochlear wall particularly in the suprastrial and tympanic portions of the spiral ligament These regions were analogous to those described previously for melanocytes in pigmented guinea pigs (LaFerriere et al 1974) In addition it has been postulated that melanocytes which are derived from neural crest cells may produce metabolic substances that are closely related to catecholamines in action (Savin 1965 LaFerriere et al 1974) Thus based on their location and possible vasomotor function injury to the albino melanocytes may secondarily affect the adjacent vasculature thereby interfering with the secretion maintenance and absorption of labyrinthine fluids

The objective of this investigation was to explore further the apparently extreme sensitivity of the melanocytes to HRP in the hope of establishing a non-toxic dose survival time that would nevertheless result in labeling of the cells It was hoped that HRP would prove to be a useful marker for melanocytes so that they could be easily identified in tissues from experimentally treated animals (as with ototoxic drugs) This would greatly aid the further study of this cell population in experiments designed to uncover their functions

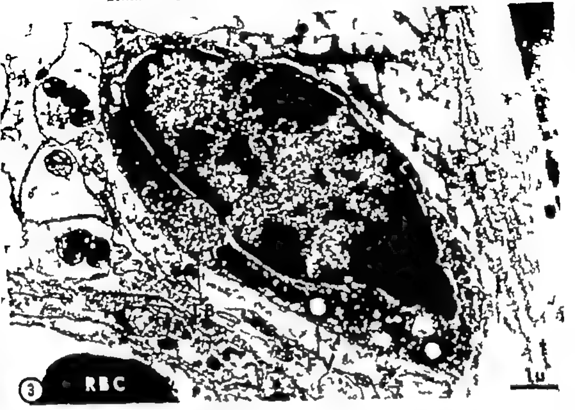

Fig. 3. A cell body of an albino melanocyte near a blood vessel in the tympanic portion of the spiral ligament after exposure to 0.005% HRP concentration (postperfusion time =1 hour). Various-sized HRP-filled vesicles (*FV*) and secondary lysosomal bodies (*LB*) were concentrated within the cytoplasm. *RBC*, red blood cell. ×1 390.

bodies and dendritic processes of the melanocytes appeared normal, although HRP-filled vesicles were scattered throughout them (Fig. 4). Ultrastructurally, endocytosized HRP was concentrated in microvesicles that were dispersed within the cells (Fig. 5).

E. 0.0005% HRP, postperfusion times <30 min

In this series of experiments, the perfused concentration (0.0005%) of HRP remained constant, but the postperfusion survival times were varied decrementally. HRP-labeling of the albino melanocytes was still clearly observed with postperfusion survival times varying from 15 min down to 5 min. However, the labeled cells were fewer in number than when postperfusion times >30 min were employed. With postperfusion survival times ≤1 min, the HRP reaction product was minimally scattered throughout the cell body of the melanocytes, but was more concentrated near their limiting cell membranes. Ultrastructurally, HRP was observed in microvesicles, no secondary lysosomal bodies were evident.

DISCUSSION

Light and electron microscopical observations of this study extend the finding of Ross et al. (1977) that perilymphatic perfusions of high concentrations of HRP can cause acute and selective injury to cochlear albino melanocytes. With concentrations of HRP >0.0005% and postperfusion survival times >1 hour, the cell bodies and dendritic processes of the al-

Fig 1 Albino melanocytes located in the suprastrial portion of the spiral ligament after exposure to a 0.5% concentration of HRP (survival time =5.5 hours) The cell body of each albino melanocyte is distended and the cytoplasm is filled with various clear vacuoles (*CV*) and HRP-filled vesicles (*FV*) Note the bulbous appearance of the dendritic processes (*arrowheads*) of the albino melanocytes ×1 105

Fig 2 An albino melanocyte located in the tympanic portion of the spiral ligament after exposure to a 0 01% HRP concentration (survival time =1 hour) The albino melanocyte was observed to contact two capillary vessels HRP-filled vesicles were observed in its cytoplasm, as well as within its dendritic processes (*arrowheads*) Note that its dendritic processes are not bulbous. ×1 105

B *0 01 % HRP*

The cochlear labyrinthine tissues appeared less stained when a 0 01% concentration of HRP was perfused and the postperfusion survival time was decreased to one hour Except for the HRP-labeled albino melanocytes HRP reaction product was absent in the suprastrial and tympanic regions of the spiral ligament

The majority of labeled albino melanocytes were located adjacent to venules in the tympanic portion of the spiral ligament The cell bodies and dendritic processes of the melanocytes appeared less distended than those cells exposed to greater concentrations of HRP HRP filled vesicles were observed within the cytoplasm and in the dendritic processes of the cells (Fig 2)

C *0 005 % HRP*

The perilymphatic perfusion of a 0 005% concentration of HRP (postperfusion survival time =1 hr) resulted in little staining of the cochlear tissues However the HRP-labeled albino melanocytes were still identifiable by light microscopy the majority of the melanocytes were located in the tympanic portion of the spiral ligament Melanocytes were observed also in the suprastrial and poststrial portions of the spiral ligament The cell bodies of the melanocytes were not distended nor were their dendritic processes bulbous Ultrastructurally the cell bodies contained HRP vesicles of various sizes as well as secondary lysosomal bodies rimmed with HRP reaction product (Fig 3)

D *0 0005 % HRP*

A perilymphatic perfusion of a 0 0005% concentration of HRP (postperfusion survival time =30 min) resulted in HRP labeling of albino melanocytes but in only minimal staining of the remaining cochlear tissues The albino melanocytes were still clearly observed along the vasculature of the cochlea. The cell

melanocytes was maintained the cell bodies were not distended and the cells appeared to be packaging the endocytoalzed HRP

The HRP-labeled albino melanocytes were located in regions of the cochlear labyrinth (i.e along the vasculature of the suprastrial tympanic and the tympanic crest regions of the spiral ligament near the vas spirale and within the osseous spiral lamina) similar to those described earlier by Ross et al (1977) In addition we observed albino melanocytes in the poststrial portion of the spiral ligament The dendritic processes of most albino melanocytes appeared to contact adjacent blood vessels walls. It was not unusual to observe the dendritic processes of one melanocyte contacting the walls of two adjacent blood vessels This anatomical relationship between albino melanocytes and the cochlear vasculature is apparently analogous to that shown between the melanocytes and blood vessels in the cochlear and vestibular labyrinths of pigmented guinea pigs (LaFerriere et al 1974)

Hilding & Ginzberg (1977) have suggested that the intermediate cells of the stria vascularis of pigmented and albino rats are in fact melanocytes based on their histochemical and comparative anatomical results We did not observe HRP uptake by any strial cell type under our experimental conditions. Presumably the absence of HRP in our experimental strial tissue was due to the existence of a perilymph–endolymph diffusion barrier (Nadol 1979 Tonndorf et al 196 Duvall & Sutherland, 197 Duvall et al 1971 and others) Winther (1971) and Duvall & Sutherland (1977) did report the uptake of HRP by strial intermediate cells (melanocytes) when HRP was intravascularly injected in albino and pigmented guinea pigs.

The reason for the extreme sensitivity of the melanocytes to HRP is unclear Type VI HRP (Sigma) contains two basic isoenzymes (Shannon et al 1966) and therefore bears a cationic charge It has been postulated that the binding of basic substances (i.e. polycations) to anionic sites on cell membranes alters their surface charge thereby causing increased cellular permeability and loss of osmotic equilibrium (Quinton & Philpott, 1973 Seiler et al 1975) Perhaps high concentrations of basic Type VI HRP may also alter the membrane permeability of the albino melanocytes and thus cause cell injury In an ancillary experiment to explore this possibility we perfused Type VIII HRP (Sigma) containing purified acidic isoenzymes (Shannon et al 1966) through the perilymphatic space Surprisingly we found albino melanocytes to be equally affected by the acidic Type VIII HRP

It is clear that further work on this subject should be carried out because of its possible relevance to understanding the mode of action of various ototoxic substances on inner ear tissue Ototoxic aminoglycosides are polycationic and have been shown to have an affinity to bind with melanin granules in vitro (Lindquist 1973) Certain local anesthetic agents (e.g lidocaine) also bind with melanin and appear to be ototoxic (Englesson et al 1976 Rahm et al 1962 Lyttkens et al 1979) The active molecular form in the case of lidocaine (and related local anesthetic agents) is thought to be cationic (see discussion in Goodman & Gilman 1975) and the anesthetic effect is said to result from the combination of the cationic form with receptors in the neuronal membrane This blocks sodium channels and prevents the propagation of nerve action potentials Thus it remains possible that some ototoxic substances including HRP in high concentrations act primarily upon the surface membranes of the melanocytes (see also Schacht et al 1977) to alter their permeability even though the precise mechanism is currently unknown.

The question of whether or not the presence of pigment granules in melanocytes by virtue of their capacity to bind with ototoxic drugs causes greater susceptibility of pigmented animals to hearing impairments is unresolved. This will be an interesting question

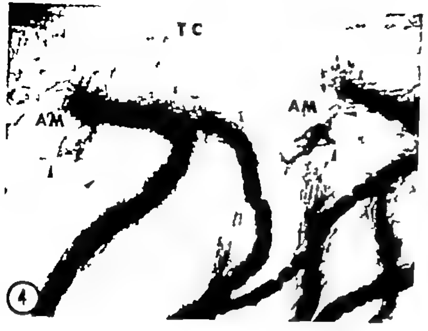

Fig 4 A surface preparation of the spiral ligament after exposure to a 0 0005 % concentration of HRP (postperfusion survival time=30 min). Two albino melanocytes (*AM*) along the vasculature beneath the tympanic crest region (*TC*) of the spiral ligament are shown The cytoplasm of these albino melanocytes contained numerous HRP-filled vesicles. The dendritic processes (*arrowheads*) appeared non-bulbous, but did contain HRP-filled vesicles ×615

bino melanocytes were markedly distended cytoplasmic vacuolization and ruptured plasmalemmas of the melanocytes were observed In contrast albino melanocytes appeared to suffer little or no injury when exposed to 0 0005 % concentrations of HRP and postperfusion survival times ≤1 hour Under these conditions the structural integrity of the

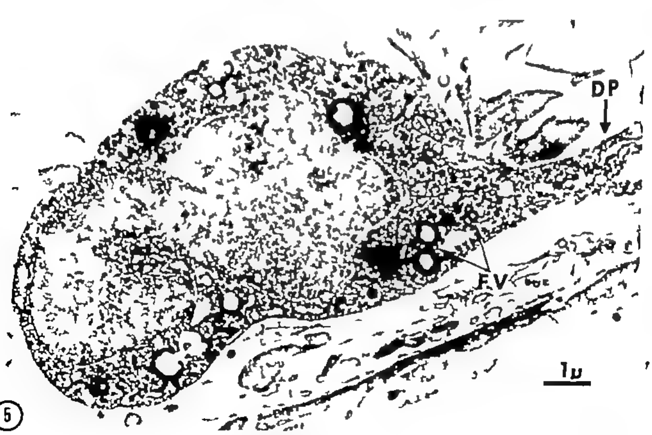

Fig 5 An albino melanocyte located in the tympanic portion of the spiral ligament after exposure to 0 0005 % concentration of HRP (postperfusion survival time=30 minutes) The indented nucleus of the melanocyte was surrounded by many cytoplasmic HRP filled microvesicles (*FV*) HRP bound vesicles were observed also in its dendritic process (*DP*). ×11 130

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Kyle E. Rarey
Dept of Anatomy
University of Michigan
Medical Science II
Ann Arbor
MI 48109
USA

to continue to explore because some experimental evidence indicates that albino and pigmented animals are affected by ototoxic drugs nearly identically (Harpur & D Arcy 1976) We have preliminary anatomical data to indicate that melanocytes from albino and pigmented guinea pigs are equally sensitive to HRP (Rarey & Ross unpublished results)

It would appear from our findings that HRP will prove to be of interest not only as another demonstrated ototoxic agent when employed in sufficiently high doses but will also be highly useful as a marker for melanocytes when administered in minute non toxic concentrations The histochemically produced HRP reaction product is visible ultrastructurally and would allow the identification of melanocytes when the effects of other substances upon them are under investigation Presumably this could lead to further clarification of the functional significance of the melanocytes with respect to microhomeostasis in the inner ear

ZUSAMMENFASSUNG

Die Wirkungen von niedrigen Konzentrationen (0,5–0,0005 %) von HRP und unterschiedlichen nach Perfusionen Überlebungsdauer (5,5 Stunden – 15 Sekunden) an Melanozyten der Albino Meerschweinchen-Cochlea wurden lichtmikroskopisch und elektronmikroskopisch untersucht Ungefähr 50 µl unterschiedlicher Konzentrationen von HRP aufgelöst in künstlicher Perilymphe wurden perfundiert durch den perilymphatischen Raum bei 32 Albino Meerschweinchen Die Gewebe wurden nachträglich histochemisch behandelt, um HRP Reaktionsprodukte zu demonstrieren. Die Resultate weisen darauf hin daß Konzentrationen von HRP >0,0005 % in Kombination mit Überlebungsdauer >1 Stunde akute und selektive Verletzungen in den Albino-Melanozyten hervorrufen zytoplasmische Vakuolisierung und Bruch der Zellmembranen kamen or Die strukturelle Integrität der Albino-Melanozyten schien unverändert, nachdem sie für ≤1 Stunde den 0 0005 %igen Konzentrationen von HRP ausgesetzt wurden Eine scheinbar nichttoxische 0,0005 %ige HRP Konzentration mag ein Mittel ergeben womit man die Melanozyten der Schnecke markieren kann wenn die Wirkungen anderer Substanzen untersucht werden.

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The efferent neurons, contralateral to the crista ampullaris we studied were stimulated electrically Electric monophasic impulses of 0 1 ms duration and 4 V strength were delivered at 200 Hz for 80 ms This stimulation is stopped 20 ms before the mechanical stimulation of the crista ampullaris Stimulating electrodes were bipolar 100 μm diameter silver wires They were lowered by stereotaxy at a 50° angle into the contralateral efferent nuclei. Recording electrodes were formed of two coupled 50 μm diameter silver wires Their bare bent point hooks the lateral canal nerve. In order to test the influence of the contralateral labyrinth we stimulated, in three experiments the contralateral lateral canal nerve using the above described parameters

RESULTS

Following a brief stimulation (0 5 ms) of the lateral canal crista, caused by an ampullopetal liquid flux (0 04 μl) a bimodal action potential appears on the lateral canal nerve Its two components have been termed N1 and N2 (Fig. 1A1 D1)

(A) *Inhibitory Responses*

1 *Contralateral efferent neuron stimulation*

In most cases stimulation of contralateral efferent neurons leads to a decrease in the amplitude of the vestibular action potential (VAP) (Fig. 1A2) compared with the control recording (Fig 1A1)

A Student s *t* test made on the differences between the N1 and N2 non-computed average values under normal conditions and after efferent system stimulation is not significant for N1 ($P<0.1$) but is significant for N2 ($P<0.01$) In extreme cases these differences can reach 20% of the amplitude in the N1 potential and 30% in the N2 potential. Efferent system stimulation also causes the appearance of an extended positive wave which follows the N2 potential (Fig. 1B2) and which is absent under normal conditions (Fig. 1B1)

In certain experiments stimulation of the efferent system has very little effect. A Student s *t* test carried out after these experiments showed no significant difference

2. *Contralateral lateral canal nerve stimulation*

Stimulation according to the same parameters as those used for the efferent neurons reduces the N2 potential considerably (Fig. 1C2) In view of the very small number of experiments (3) statistical tests have not been made

(B) *Facilitating Responses*

Generally speaking when efferent neuron stimulation did not seem to have any influence on the VAP we observed that the computer averaging of 10 responses revealed an increase in the N1 and N2 potential amplitude (Fig. 1D2) compared with the averaging of 10 responses obtained under normal conditions (Fig 1D1) The N1 potential amplitude increases more than does the N2 potential We have also observed an increase in the amplitude of the potentials consecutive to N1 and N2 (Fig. 1D1–2)

DISCUSSION

In a forthcoming article (Sans & Dechesne 1979) we submit the hypothesis that the N1 and N2 potentials are caused by the bringing into play of the two types of vestibular sensory cell. In these conditions, influence of the efferent system could be interpreted differently according to whether its stimulation brings about inhibitory or facilitating responses

Inhibitory responses

Efferent neuron stimulation The N2 potential decreases more than does N1 potential Dechesne... Goldberg...

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CONTROL OF THE VESTIBULAR NERVE ACTIVITY BY THE EFFERENT SYSTEM IN THE CAT

C Dechesne and A Sans

From Laboratoire de Neurophysiologie Sensorielle Université de Montpellier II Montpellier Cédex France

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Abstract The effect of vestibular efferent system stimulation on the vestibular action potential (VAP) recorded after brief mechanical ampullopetal stimulation of the lateral canal crista was studied in the cat. When an inhibitory action was recorded it had an effect on the VAP component N2 potential and was also characterized by a positive post potential When stimulation seemed to have no effect, computer averaging showed a weak facilitating action affecting the N1 potential The hypothesis of a double efferent system is discussed

Sensory cell activity of mammal vestibular receptors is controlled by efferent fibres which establish different synaptic contacts with the two types of hair cells The efferent neurons form axo-dendritic synapses with the chalice membrane which afferents type I cells and axo-somatic synapses with the plasmic membrane of type II cells Recent anatomical studies on mammals using HRP retrograde transport have located the cell bodies of these neurons (Gacek & Lyon 1974 Warr 1975 Raymond 1977)

However there are few published physiological studies concerning the mode of action of the efferent vestibular system These studies indicate an essentially inhibitory action (Ledoux 1958 Sala 1965 Rossi et al 1978) and seem to be confirmed by work carried out at the receptor level of the lateral line in the *Lota lota* fish Flock (1973) Flock & Russell (1973) have recorded IPSP as well as an inhibition of spontaneous activity of the primary afferents after efferent system stimulation However excitatory responses have been obtained in the cat by Sala (1965) and considered in the frog by Gribenski & Caston (1976) Some authors believe that the efferent system plays only a very small part (Dieringer et al 1977) if any part at all (Keller 1976) in the control of afferent activity

In this work we have tested the effects of electrical stimulation of efferent neurons on the vestibular nerve action potential (VAP) recorded after short selective mechanical stimulation of the lateral canal A preliminary note (Dechesne & Sans 1979) describes this method of stimulation

MATERIALS AND METHODS

Nineteen adult cats were used in this first study They were anesthetized with an intraperitoneal injection of pentobarbital (40 mg/kg) to maintain the level of anesthesia subsequent doses were given through a cannula placed in the saphenous vein The animals were mounted in a stereotaxic frame In order to expose the semicircular lateral canal the animal was laid on its side A steel needle (0 25 mm exterior diameter) was introduced into the membranous lateral canal This needle was connected to a 1 μl syringe via a polyethylene catheter this apparatus was filled with endolymphatic liquid The syringe plunger was set into motion by an electromagnetic device at a speed allowing the injection of 0 115 μl/ms

facilitating and would control presynaptically type II cell activity

This hypothesis can be compared to phenomena observed at the cochlear level The cochlear potential has several components. the microphonic potential (CM) the summating potential (SP) and the bimodal action potential with its two components N1 and N2. Electrical stimulation of the efferent system in the young cat causes an SP as well as an N2 increase and a N1 decrease (Carlier & Pujol, 1976) According to Desmedt (1975) post synaptic innervation of the inner hair cell seems to be responsible for N1 decrease presynaptic innervation of outer hair cells would be the cause of SP increase

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ZUSAMMENFASSUNG

Die Wirkung der Stimulation des efferenten Vestibular Systems auf das vestibuläre Aktionspotential (VAP) nach kurzer ampullopetaler Stimulation der lateralen Crista wurde an der Katze studiert. Eine inhibierende Aktion wurde hauptsächlich auf die N2-Komponente des vestibulären Aktionspotentials (VAP) festgestellt Diese Inhibition ist auch durch ein positives Post-Potential ausgezeichnet. Wenn die Stimulation ohne Wirkung scheint, zeigt die Durchschnittberechnung auf Komputer das Auftreten einer schwachen das Potential N1 anregenden Aktion. Die Hypothese des Bestehens eines doppelten efferenten Systems wird in der Diskussion erörtert.

RÉSUMÉ

On a étudié chez le chat l'effet de la stimulation du système efférent vestibulaire sur le potentiel d'action vestibulaire (VAP) après stimulation ampullopète brève de la crête latérale On a constaté une action inhibitrice essentiellement sur la composante N2 du VAP Cette inhibition se caractérise également par un post-potentiel positif Lorsque la stimulation paraît inefficace le moyennage sur computeur montre qu'il existe une action excitatrice faible qui porte sur le potentiel N1 L'hypothèse de l'existence d'un double système efférent est envisagée dans la discussion

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C Dechesne M.D
Laboratoire de Neurophysiologie Sensorielle
Université de Montpellier II
34060 Montpellier Cdx
France

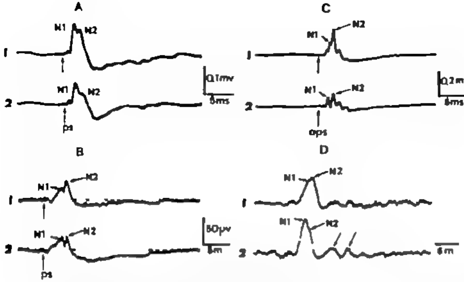

Fig 1 Effects on the vestibular action potential of contralateral efferent neurons stimulation (A2–B2–D) or of the contralateral lateral canal nerve stimulation (C2) A1–B1–C1 and D1 are control recordings of the vestibular action potential with its two components N1 and N2. Note in A2 the considerable fall in N2 potential in C2 the decrease in N2 after contralateral nerve stimulation, in D2 the increase in N1 potential and consecutive potentials (*arrows*) The recordings in D are the result of the averaging of 10 responses. aps, ampullopetal stimulation

Thus the inhibitory system in particular would have an effect on type I cells which might then be responsible for the N2 potential type II cells being responsible for N1 potential Positive waves following the N2 potential (Fig 1 B2) can be interpreted as a sign of a prolonged inhibition of the vestibular receptors and thus of a decrease in the spontaneous activity existing on their afferents

Ampullar contralateral nerve stimulation Recording of inhibitory responses shows that a reciprocal labyrinth control exists This inhibitory control primarily affects the N2 potential and could be transmitted by the efferent system

In 1958 Ledoux was already inhibiting the homolateral afferent discharge with caloric stimulations of the contralateral labyrinth Likewise Schmidt demonstrated in 1963 the fact that ampullar stimulation caused an efferent response on the contralateral vestibular nerve this effect being more significant between homologous receptors

Facilitating responses

Contrary to the phenomenon which occurs during inhibitory responses the N1 potential amplitude increases while N2 potential is hardly affected These responses could be the manifestation of a rebound in afferent activity following an inhibition This inhibition would take place during the period which separates the electrical stimulation of the efferent system from the mechanical crista stimulation (70 ms). However this hypothesis does not explain why the N2 potential which is the most affected by the inhibition is not also the most affected by a phenomenon of facilitatory rebound

In such cases it would be better to invoke the existence of a facilitating efferent system mainly controlling type II cells responsible for the N1 potential The increase in the amplitude of potentials consecutive to the VAP (Fig 1 D2) would be a consequence of this facilitating effect Sala (1965) also recorded excitatory responses in the cat and put forward the hypothesis that the efferent system could play an excitatory or inhibitory role according to the functioning state of the receptors

Thus two different efferent systems could exist One would be inhibitory and would have a postsynaptic control on the nerve chalice membrane in type I cells The other would be

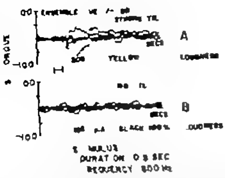

Fig. 1. Comparison of vestibulo-spinal reflex and auditory loudness sensation responses of two eighth nerve electrodes. The stimulus duration was 500 msec and frequency was 500 Hz. For this and subsequent figures, *Abscissa*: time in seconds. *Ordinate*: torque in newton-meters. Computer-averaged responses were obtained on five successive presentations of the stimulus. The average of these responses ±1 S.D. were plotted by the computer as a function of time. (A) Yellow "Y" electrode. Stimulus bar under top trace begins at one second after onset of recording and indicates stimulus duration. (B) Black "B" electrode. Stimulus current amplitude: 200 μa for yellow electrode, 185 μa for black electrode. Vestibular sensations are recorded at the upper right and auditory loudness (scaled as percent) are recorded at the lower right corner of each trace.

METHODS

Data acquisition and analysis

The subject's postural responses were recorded using a piezoelectric force platform interfaced to a LSI 11 microprocessor which was in turn connected hierarchically to a PDP 11/40 minicomputer (Black et al. 1977*a*). The patient was placed on the platform and instructed to perform the Jendrissik maneuver. This instruction has the effect of simulating as much as possible an inverted pendulum. All recordings were performed with the subject's eyes closed and his head facing forward (Nashner & Wolfson 1974). The instantaneous center-of-force was calculated 10 times per second using moments. This center-of-force calculation was zeroed immediately before any stimulation to reduce response variability. (All human subjects are in constant motion while standing.) The torque exerted by the subject on the force platform during the stimulation was calculated by multiplying the Y-plane (right–left) (Hixson et al. 1966) projection of the center-of-force by the subject's weight. Computer averaged responses were obtained on five successive presentations of the stimulus. The average of these responses ±1 S.D. were plotted by the computer as a function of time.

For evaluation of the recorded response the subject was assumed to have an "objective tilt" when the mean value of the data was more than one standard deviation (S.D.) away from "zeroed" (prestimulus level) for a period of at least 0.25 sec.

Electrical stimulus pulse trains were presented to the subject's implanted electrodes using 0.5 msec current balanced biphasic pulses at either 300 or 500 Hz, for 0.1–2 sec. Single pulse and low pulse repetition rates did not cause observable vestibular effects. Slower pulse rates caused "full or stuffy" sensations.

Maximal stimulation limits were defined by the following sensory maxima.

(*a*) Auditory loudness scaling (threshold to "too loud"–0–100%). The patient had been taught by one of us (FBS) to scale the loudness of his auditory responses (Simmons et al. 1979).

(*b*) Vestibular system, a strong sensation of head turning or tilt which the subject determined would cause him to fall or would require moving his feet in order to maintain upright stance.

(*c*) Sensation of maximal "pressure", i.e. if the pressure were to become more severe it might cause pain. At no time during the procedure was the sensation of pain elicited.

RESULTS AND DISCUSSION

While pulses to all four electrodes produced auditory sensations, only two had postural effects. Of these two, stimulation of the "yellow" electrode produced responses at lower currents and was also responsible for the "pressure" sensations. Auditory thresholds were obtained at less than 20 nanocoulombs

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HUMAN VESTIBULO-SPINAL RESPONSES TO DIRECT ELECTRICAL EIGHTH NERVE STIMULATION

F Owen Black F Blair Simmons and Conrad Wall III

From the Raymond E. Jordan Human Vestibular Testing Laboratory Department of Otolaryngology Eye and Ear Hospital Unive sity Health Center of Pittsburgh Penn USA

(Received July 11 1979)

Abstract Left right (y-axis) vestibulo-spinal torque re sponses were recorded from two of four intramodiolar stimulus electrodes placed for auditory stimulation in one human subject For both electrodes shorter (100 msec) stimuli caused either subjective or objective head turning whereas stimuli lasting longer than 0.5 sec caused either subjective or objective body tilt depending upon the elec trode stimulated. The two electrodes tested also showed strikingly different auditory and vestibular effects Stimulation of the remaining two electrodes caused no detectable vestibulo-spinal responses

A previous evaluation of subjects implanted with single electrode scala tympani cochlear stimulators indicated that some patients also experienced vestibular stimulation (Black 1977*a b* 1978) A patient who had undergone multiple electrode intramodiolar cochlear nerve implantation experienced postural displacement upon stimulation of two of his four electrodes This study was undertaken in order to examine quantitatively his postural sway responses to direct electrical stimulation of his eighth nerve

The patient was a 58-year-old male with total deafness from neomycin ototoxicity Elec tronystagmography performed prior to the implant procedure showed a direction fixed (right) positional nystagmus with a bilaterally reduced (right>left) response to caloric irriga tion with a strong right directional preponder ance Optokinetic responses were symmetri cal The patient underwent a four-electrode intramodiolar cochlear implant in the right ear on August 22 1977

Early in the post implantation experiments the subject noticed a sensation of fullness in his ear when one of the four electrodes was stimulated This fullness began at about 3 dB below perceptual auditory threshold. He also noted that except near threshold sounds produced tended to have "unpleasant" or "annoying" qualities which were never encountered while stimulating the other three elec trodes He also observed that certain pitches make me tilt The patient did not have a sense of rotation nor did he have a sense of falling—just a sensation of tilt or staggering. The subject experienced postural unsteadiness only during electrical stimulation He had no previous spatial disorientation sensations similar to those experienced The use of the stimulator did not otherwise limit his physical activities

The subject had no measurable hearing The responses obtained were attributed to tactile sensations The right tympanic membrane was thickened but mobile The otologic examination was otherwise normal The subject had a slight resting tremor which probably could be related to a history of smoking more than two packages of cigarettes a day (nicotine tremor) The cranial nerves and vibration senses were intact and motor coordination function was normal upon physical examination

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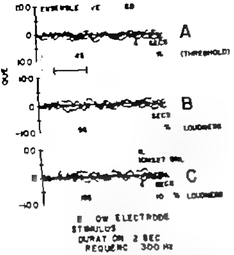

Fig. 4. Lower frequency (300 Hz) longer duration (2 sec) stimulation of the yellow electrode resulted in less tilt but greater auditory sensation at relatively less current amplitudes (cf. Fig. 2 and 3).

the "yellow" electrode is in the inferior division of the vestibular nerve (see also Suzuki et al. 1969). Note also that the major postural disturbance occurred after the stimulus was discontinued and persisted for several seconds (also see Fig. 3). A similar force platform response was noted by Nashner & Wolfson (1974) in response to galvanic stimuli.

Stimulus duration was an important determinant of the vestibular response. Brief pulse trains (0.1 sec) caused only momentary or brief head turning to the left at the onset of the stimulus. Longer trains (2 sec) caused subjective and objective body tilting and no head rotation. Fig. 2 illustrates this difference at three stimulus intensities for 0.1 sec pulses. Only the highest (325 μa) (Fig. 2C) stimulus current produced an observed effect (head turning) in association with a minimal objective body tilt (Fig. 2C). Note also that the change in reported loudness with change in stimulus magnitude is less for the brief-pulse stimulation. This phenomenon is consistent with both normal psychophysical auditory perception and with other observations on electrical stimulations. Longer duration (2 sec) stimulus trains (Fig. 3) produced sustained subjective and recorded tilts but no head turning. Two sec. 500 Hz stimuli produced lower thresholds for subjective as compared to recorded tilts.

Pulse rates below about 300 Hz reached maximum tolerance loudness without producing a measurable force platform response. Fig. 4 shows this effect at 300 Hz, 2-sec stimulation of the "yellow" electrode. Maximum auditory loudness sensation was reached before subjective vestibular thresholds were reached. The maximum tolerable stimulus current (Fig. 4C) at this frequency was 155 μa which was approximately the midrange response for 500 Hz stimulation.

Fig. 5 shows the response of the "black" electrode for stimulus durations of 2 sec at 500 Hz for comparison with the "yellow" electrode responses (see Fig. 3). A marked sensation of tilt was perceived by the patient but significantly less objective tilt was recorded by stimulation of the "black" electrode. A maximum tolerable sensation of tilt was achieved before measurable vestibulo-spinal tilt thresholds were reached.

Table I summarizes the results of these experiments. The "yellow" electrode was more sensitive than the "black" by both objective recordings and subjective tilt response esti-

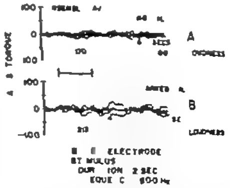

Fig. 5. Longer duration stimulation of the black electrode results in tilt sensations, but no significant objective tilt and no head turning. Auditory loudness sensation did not change significantly with stimulus duration for the black electrode.

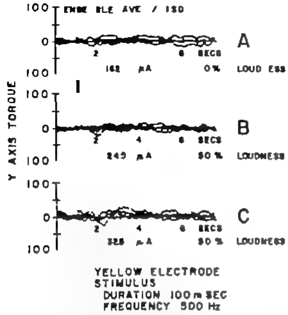

Fig 2 Effect of stimulus amplitude for short duration electrical stimuli upon vestibulo-spinal reflexes. Only minimal tilt resulted from very high current (325 μA) amplitudes (100 msec 500 Hz stimuli) presented to the most tilt sensitive ("Y") electrode For these stimulus conditions a relatively greater head turning (vestibulo-collic reflex) also occurred but could not be accurately estimated by the force platform technique used in this study

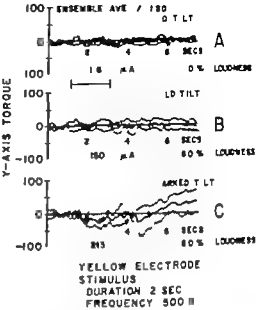

Fig 3 Longer duration (? sec) stimuli presented to the yellow electrode at 500 Hz yielded relatively greater tilt than auditory loudness sensations with lower stimulus amplitudes (cf Figs 2 and 3)

for both electrodes The "maximum loudness es were obtained at 40 and 45 nanocoulombs when using pulses of 1 25 msec [1] The stimulus magnitudes measured during the to-be re peated observations here do not agree with this much larger body of data and are therefore suspect We have chosen to use them for lack of a better index The reader should however understand that while they are internally con sistent absolute μa values may be inaccurate

Fig 1 illustrates the relative sensitivity of the two tilt sensitive electrodes Stimulation of the "yellow electrode (Fig 1 A) at only 60% maximum loudness produced the maximum tilt sensation that the subject was willing to tolerate The subject described his sensations as a startle which corresponded in time with the large tilt response variability recorded be tween 2 and 3 25 sec The black electrode (Fig 1 B) on the other hand created neither subjective nor objective tilt when stimulated at 100% of maximum loudness The stimulus current for the black" electrode was 7% less than for the yellow electrode

These consistently observed results suggest that the electrode location determines stimulus response to electrical activation of the eighth nerve and that there is little or no stimulus spread for the stimulus intensities employed (Tokamasu et al 1971) Electrical stimulation of vestibular nerve branches in the cat and monkey result in characteristic vestibulo-ocular and vestibulo-spinal reflex responses (Suzuki et al 1969) No such direct electrical stimulation data exist for man Neuroanatomical connections of the vestibular system are thought to be basically similar in cat monkey and man (Brodal 1969) Assuming valid cross species interpolation on this neuroanatomical evidence and based upon spatial anatomical relations of the surgical approach (Simmons et al 1979) probable location of

[1] Multiplying these numbers by four yields the approximate values in μa s. Note that the pulses used in this study were 0.5 msec (per phase), ×4 longer Comparable threshold-to-maximum loudness data are not available for these pulses.

employed pulse trains without a DC component. However the tilt behavior for the both externally applied DC and internal pulses seem very similar in both subjective and objective frames (Njiokiktjien & Folkerts 1971) This may be due to the coincidence of the body s limited response repertoire to such stimuli, or one of many other alternatives Whatever the mechanism it seems clear that nerves from gravity receptors probably the saccular nerve fibers were predominantly affected in the subject under study At no time were eye movements observed in response to electrode stimulation Thresholds for body sway are however lower than for galvanic induced nystagmus (Coats 1973) It is unlikely that stimulus current spread was more than about 0.2 mm from the electrode tips (McNeal & Teicher 1977 Markham & Curthoys 1972)

The fact that objective manifestations were observed only at very high stimulus rates argues strongly that nerve tissue excitation occurred only at "tetanizing" rates—rates which invade the relative refractory periods of neurons and thereby do cause an oscilloscope—apparent DC shift in the baseline recordings from these neurons This effect is not limited to the site of stimulation but can affect the pre and post-synaptic potentials at the next synapse (Eccles 1964 Searles & Barnes 1977) However both electric field potential (galvanic) and intracellularly injected electric current are thought to modulate spike trains in a manner similar to motion stimuli (Kirsten 1975) The concept of a galvanic induced DC bias is also compatible with the observation that interruption of a galvanic current resulted in tilt responses equivalent to initiation of a response in the opposite direction (Nashner & Wolfson, 1974)

The major objective response (tilt) probably resulted as much or more from disruption of the ongoing spontaneous discharging of vestibular fibers as from the centrally-transmitted (and presumably more immediate) effects of the shocks themselves Measurable tilts began at about 0.5 sec after stimulus onset, even for 0 1 sec stimuli. Postural homeostasis was not restored for many seconds afterwards—an effect which may or may not be blamed on the electrical shocks

Finally the question of most pragmatic importance is the possible limitation of the use of cochlear stimulation by vestibular side effects. This is the second report that such effects can occur (Black et al. 1978) Current information is inadequate to make any judgment either on the incidence or trade-offs for usable hearing vs slight balance anomalies In this case report the use of the "yellow" electrode is at this time limited more by the "unpleasant" quality of the "electrical sounds" than by either subjective or objective vestibular effects *if* pulse rates are below 300 Hz. We need more information Indeed we hope this report will encourage other experimenters to assess their stimulation results which are pertinent to evaluating this problem.

These results also bring up the possibility that sustained (long duration) electrical stimuli which would be required for auditory stimulation tend to yield caudal vestibulo-spinal reflex responses instead of vestibulo-collic responses It should be emphasized that not all electrical stimuli to the labyrinth and eighth nerve yield vestibular responses (Kimm et al., 1979)

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ZUSAMMENFASSUNG

Links-rechts (y-Achse) vestibulo-spinale Drehmomentreaktionen wurden von zwei unter vier intramodiolaren Reizelektroden aufgezeigt, die zur Gehörstimulation bei einem Menschen angebracht waren. Bei beiden Elektroden verursachten kürzere Reize (100 msek) entweder subjektives oder objektives Kopfdrehen, wohingegen Reize, die länger als 0,5 Sekunden dauerten, entweder subjektive oder objektive Körperneigung verursachten, abhängig von der jeweils stimulierten Elektrode. Die beiden getesteten Elektroden zeigten auch auffallend unterschiedliche Gehör- und Vestibularisstörungen. Stimulation der verbleibenden zwei Elektroden verursachte keine feststellbaren vestibulo-spinalen Reaktionen.

Table I

Stimulus			Response				
			Tilt		Turning		
Electrode Y=yellow B=black	Strength (μa)	Duration (sec)	Subjective	Objective	Subjective	Objective	Fig.
Y	200	0.5	Yes	(Jolt)	–	–	1A
B	185	0.5	No	–	–	–	1B
Y	162	0.1	No	–	No	–	2A
	245	0.1	–	–	–	–	2B
	325	0.1	–	Mild	–	–	2C
Y	116	2.0	No	No	–	–	3A
	150	2.0	Mild	No	–	–	3B
	213	2.0	Marked	Yes	–	–	3C
Y	45	2.0	No	No	–	–	4A
	98	2.0	No	No	–	–	4B
	155	2.0	Yes	No	–	–	4C
B	213	0.1	No	No	Slight	–	None
	275	0.1	No	(Jolt)	Strong	–	None
B	170	2.0	No	No	No	–	5A
	213	2.0	Yes	(Jolt)	No	–	5B

mates With stimulation of the "yellow" electrode short duration (100 msec) stimuli caused an impulse type response as recorded on the force platform which the patient described as a "jolt" Increasing the stimulus duration to two seconds caused both subjective and objective tilt when the stimulus was above a threshold value The subjective was somewhat lower than the objective tilt threshold

When stimulation was delivered to the "black" electrode there was no objective tilt as measured on the force platform It was however possible to record a subjective response either in head turning or tilt when the stimulus value was increased substantially above threshold The significant parameter in differentiating between tilt and head turning in this case was also stimulus duration The post stimulus destabilization did not persist as long for the "black" electrode as for the "yellow" electrode

COMMENT

Results of this experiment indicate that direct electrically induced auditory vestibular stimulation is a complex interaction between the electrode location stimulus frequency stimulus duration and stimulus amplitude In this instance the "yellow" electrode seemed closer to viable vestibular fibers than auditory fibers *re* perceptual thresholds In spite of this the auditory threshold sensitivity and suprathreshold perceptions and difference limens were not quantitatively different from other stimulated electrodes which produced no vestibular effects Vestibular effects of "black" electrode stimulation were very minor and were typically unnoticed by the subject during many hours of sound stimulation testing. In fact one of us (FBS) was completely unaware of a vestibular effect on the "black" electrode before these experiments nine months after the electrodes were placed

This is the first time that direct electrical stimulation of the vestibular nerve has been examined in the human The results presented above cannot be directly compared to galvanic stimuli presented by a field potential Most transcutaneous galvanic paradigms probably produce DC electrical modulation of the vestibular potentials (Fredrickson et al 1966 Lowenstein 1955 Spiegel & Scala, 1943 Vito et al 1956) The present experiment

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EFFECT OF OFF-VERTICAL TILT AND MACULAR ABLATION ON POSTROTATORY NYSTAGMUS IN THE SQUIRREL MONKEY

M Igarashi M Takahashi T Kubo B R. Alford and W K Wright

From the Department of Otorhinolaryngology and Communication Sciences Baylor College of Medicine Houston, Texas USA

(Received August 28, 1979)

Abstract The slow phase eye velocity (SPEV) and duration of post-rotatory nystagmus (PRN) were studied in squirrel monkeys (*Saimiri sciureus*) after ramp speed rotation (0–200°/sec, with 1°/sec² angular acceleration). When the results were compared between straight upright vertical rotation 9° tilt rotation and 18° tilt rotation, faster decay both in SPEV and in duration was found in the tilt rotation situations. Differences in nystagmic decay curves by tilting rotation axis could be from the convergence of maculo-semicircular canal inputs. Subsequently bilateral macular ablation (two-stage) was performed. The difference in nystagmus decay curves between three different rotations was reduced, therefore the change of gravity direction perceived through gravity receptors other than macular endorgans was minimal and did not produce a difference in three different rotations

The semicircular canal induced nystagmus can be influenced by the direction of gravity or by an application of linear acceleration (Benson 1974 Benson & Bodin 1966*a* Lansberg et al 1965 Oosterveld & Van der Laarse 1969) Morphologically the macular end organ is structured as the primary receptor of the gravitational and linear forces and neurophysiological data have shown that the macula cannot perceive angular acceleration (Goldberg & Fernandez, 1975) However the crista ampullaris—cupula system could perceive gravitational or linear force (Benson et al. 1970 Estes et al 1975) The macula and crista interactions in all animals living in the terrestrial environment have been evolved under the continuous influence of the earth's gravity force

In order to evaluate *the effect of change of* gravity direction on the semi circular canal induced nystagmus we compared post-rotatory nystagmus (PRN) in squirrel monkeys when the cephalo-caudal rotational axis was straight upright vertical and when the axis is tilted off-vertically Furthermore in those animals, we placed bilateral macular ablation in order to assess the contribution from other gravity sensors

SUBJECTS AND METHODS

Nine healthy young adult squirrel monkeys (*Saimiri sciureus*) body weight ranging between 450 to 650 g, were used in this study

The monkey was placed *in the laboratory* built restraining device and the test was begun 15 min after the injection of amphetamine 0.5 mg/kg Utilizing our *motor-driven turntable* a ramp speed rotation (*with 1°/sec² angular acceleration*) was given *to the animal* (in the dark) and it was stopped at the maximum speed of 200°/sec *For the off vertical rotations (9° and 18° tilt) exactly* the identical rotation mode was given Eye movements were re

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Dept. of Otolaryngology Tokyo Women's Medical College, Tokyo Japan

Dept. of Otolaryngology Osaka University School of Medicine Osaka, Japan

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F Owen Black M D F.A C.S
Eye and Ear Hospital
230 Lothrop Street
Room 923
Pittsburgh Pennsylvania 15213 USA

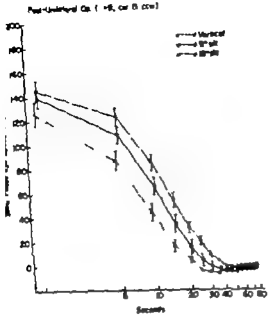

Fig. 2 Similar to Fig. 1 Post-unilateral macular ablation status

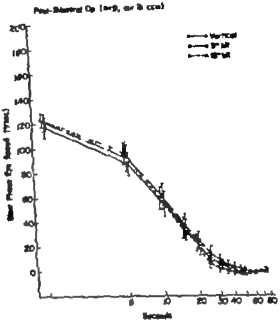

Fig. 3 Similar to Fig. 1 Post-bilateral macular ablation status.

quired to reach zero level The time constant in this condition was 10 4 sec

When the influence of tilting rotation axis to the PRN was compared between straight upright vertical 9° tilt and 18° tilt rotations no more than 15% difference existed in the maximum SPEV immediately after the cessation of the 200°/sec stimulus. The most dominant difference created by rotatory axis tilting was the shortening of the first phase duration of PRN When the PRN first phase duration time after 200°/sec stimulus in upright vertical rotation was counted as 100 it reduced to 45 after 9° tilt rotation, and it was 33 after 18° tilt rotation

II Post-unilateral macular ablation

The maximum SPEV immediately after the 200°/sec stimulus cessation was 144.8°/sec in straight upright vertical axis 139.8°/sec in 9° tilt condition and 126 9°/sec in 18° tilt (Fig and Table I) Those values were 87 6% 97 3% and 90 1% of the values obtained in pre-operative conditions respectively

PRN's first phase durations to reach zero level in straight upright vertical axis, 9° tilt axis and 18° tilt axis were 39 sec 30 sec, and 25 sec respectively and all of those were 52.0% 88.2% and 100 0% of each pre-operative duration therefore the difference between pre operative and post-unilateral operative conditions was large (48 0%) in straight vertical rotation, less (11.8%) in 9° tilt axis and none in 18° tilt axis When the time constant of decay curves were compared between pre and post-unilateral operation conditions it reduced to 75 6% of pre-operative value in the vertical axis rotation whereas it reduced 81 1% and 87.5% of pre-operative values in 9° and 18° tilt axis rotations

III Post-bilateral macular ablation

As is seen in Fig. 3 the curves of SPEV duration time in three different rotatory situations showed close approximation after bilateral macular ablation The maximum SPEV immediately after the stimulus cessation in straight vertical axis 9° tilt axis and 18° tilt axis were 120 7°/sec 117.6 /sec and 123.8°/

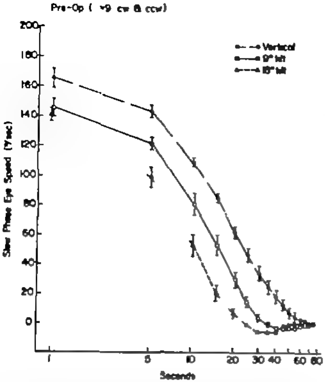

Fig 1 Comparison of the averaged slow phase eye speed decay of the postrotatory nystagmus (after the cessation of 0–200°/sec stimulus) between upright vertical 9° tilt and 18° tilt rotations in nine normal squirrel monkeys *Ordinate* slow phase eye speed (°/sec) *Abscissa* postrotation time (sec)

corded on a Beckman dynograph recorder through the DC amplifier

All squirrel monkeys received two stage bilateral utricular nerve section and saccular macula destruction Pre and post-operative testings were done about once a week For each time only one kind of test was done A minimum of three measures for each test situation were obtained pre-operatively Post operatively (both post-unilateral and post bilateral) 2–3 measures were obtained in each stimulus mode over more than two weeks period

Electro-oculographic data were analyzed manually In all situations the slow phase eye velocity (SPEV) immediately after the stimulus cessation and thereafter every 5 sec until nystagmus disappeared completely were averaged among all recordings in each stimulus mode The nystagmus after clock wise rotations and those after counter-clock wise rotations were averaged in all instances including the condition after unilateral macular ablation Thereafter the means and standard errors of the mean were calculated

RESULTS

I *Normal squirrel monkey*

The results are displayed in Fig. 1 and Table I In straight vertical rotation the maximum SPEV immediately after the cessation of 200°/sec stimulation was an average 165.3°/sec SPEV reduced gradually along the time course The PRN first phase duration time was about 75 sec after stimulus cessation the time constant (i e the time that SPEV reduces 63% of maximum value) (Robinson 1975 Waespe & Henn 1977) of this decay curve was 20 1 sec

In 9° tilt situation the maximum SPEV after the 200 /sec stimulus was an average 143 7°/sec which was 86.9% of the value obtained after straight vertical rotation The PRN first phase duration time to reach zero level was an average 34 sec after stimulus cessation The time constant declined to 14 8 sec after 9° tilt.

In 18° tilt condition the maximum SPEV immediately after the 200°/sec stimulus was an average 140 8 /sec which was 84 9% of the value obtained after straight upright vertical rotation The eye speed decline was rapid in this last case An average of 25 sec was re

Table I *Maximum slow phase eye velocity (MSPEV) and duration of PRN first phase*

	Pre op	Post unil op	Post bil. op
Vertical			
MSPEV (°/sec)	165.3	144 8	1?0.7
Duration (sec)	75	39	?2
9° tilt			
MSPEV (/sec)	143 7	139.8	117 6
Duration (sec)	34	30	40
18° tilt			
MSPEV (°/sec)	140.8	126.9	123 8
Duration (sec)	25	[illegible]	[illegible]

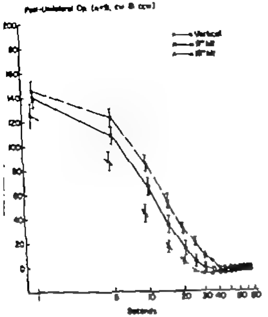

Fig. 2. Similar to Fig. 1. Post-unilateral macular ablation status.

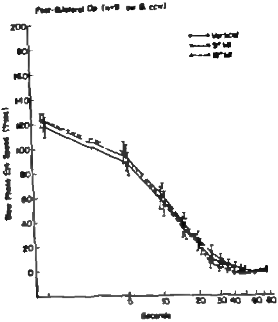

Fig. 3. Similar to Fig. 1. Post-bilateral macular ablation status.

quired to reach zero level. The time constant in this condition was 10.4 sec.

When the influence of tilting rotation axis to the PRN was compared between straight upright vertical, 9° tilt and 18° tilt rotations, no more than 15% difference existed in the maximum SPEV immediately after the cessation of the 200°/sec stimulus. The most dominant difference created by rotatory axis tilting was the shortening of the first phase duration of PRN. When the PRN first phase duration time after 200°/sec stimulus in upright vertical rotation was counted as 100, it reduced to 45 after 9° tilt rotation, and it was 33 after 18° tilt rotation.

II. *Post-unilateral macular ablation*

The maximum SPEV immediately after the 200°/sec stimulus cessation was 144.8°/sec in straight upright vertical axis, 139.8°/sec in 9° tilt condition, and 126.9°/sec in 18° tilt (Fig. and Table 1). Those values were 87.6%, 97.3% and 90.1% of the values obtained in pre-operative conditions respectively.

PRN's first phase durations to reach zero level in straight upright vertical axis, 9° tilt axis and 18° tilt axis were 39 sec, 30 sec, and 25 sec respectively, and all of those were 52.0%, 88.2% and 100.0% of each pre-operative duration; therefore, the difference between pre-operative and post-unilateral operative conditions was large (48.0%) in straight vertical rotation, less (11.8%) in 9° tilt axis and none in 18° tilt axis. When the time constant of decay curves were compared between pre- and post-unilateral operation conditions, it reduced to 75.6% of pre-operative value in the vertical axis rotation, whereas it reduced 81.1% and 87.5% of pre-operative values in 9° and 18° tilt axis rotations.

III. *Post-bilateral macular ablation*

As is seen in Fig. 3, the curves of SPEV duration time in three different rotatory situations showed close approximation after bilateral macular ablation. The maximum SPEV immediately after the stimulus cessation in straight vertical axis, 9° tilt axis, and 18° tilt axis were 120.7°/sec, 117.8°/sec and 123.8°/

sec respectively Those values were 73 0% 81 8% and 87 9% of the values obtained in the pre-operative conditions respectively Duration of the PRN first phase was 52 sec 40 sec and 38 sec respectively and those values were 69 3% 117 6% and 152 0% of those values obtained in the pre-operative condition the duration after off vertical rotations increased when compared to those obtained in the pre-operative stage

When the PRN first phase duration time in straight vertical axis was counted as 100 it was 45 in 9° tilt and was 33 in 18° tilt before the macular ablation After unilateral macular ablation those values were 100 77 and 64 and post-bilateral macular ablation those were 100 77 and 73 respectively The difference in PRN duration time between three test conditions vertical axis 9° tilt axis and 18° tilt axis decreased after macular deafferentation A similar condition was found when the time constant was compared After the bilateral macular ablation these were 13 3 12 7 and 11 5 sec at vertical 9° and 18° tilt axis rotations respectively

Table I displays the difference in maximum SPEV and duration of PRN As far as the influence of the surgery to the PRN is concerned the largest change was observed after the rotatory axis was upright vertical A difference was clear between the result obtained after unilateral operation and bilateral operation in the situation of straight vertical axis rotation however when the rotation was given in 9° or 18° tilt axis the difference was less prominent

The secondary phase nystagmus (direction opposite) was seen in pre-operative and post unilateral operative status mostly in tilt rotation situations The complete disappearance of nystagmus including both first and second phase was 60–75 sec

IV *Statistical analysis*

The analysis of variance (repeated measures) was used first to investigate the main effect of operational conditions (collapsing all data among the tilt conditions and nystagmus decay conditions) The difference was found statistically significant ($p<0\ 05$) When the main effect was studied between tilt angles the difference was found to be statistically significant ($p<0\ 01$)

When the simple effect was compared between pre and postunilateral operation (collapsing data between different tilt angles and nystagmus decay conditions) the difference was not statistically significant ($<0\ 1$). On the other hand when the pre-operative state was compared to post-bilateral operation condition the difference was found to be statistically significant ($p<0\ 05$) and therefore confirmed that the manifestation of macular ablation effect is only dominant after the bilateral total ablation

V *Systemic response*

Even though this off vertical rotation is a motion sickness inducing stimulus for man squirrel monkeys in the present series did not exhibit any clear emesis during and after the off vertical rotation however many animals showed excess saliva retching and chewing which might indicate prodromal signs Also many animals showed floundering reactions, which might indicate that animals were suffering from unpleasant sensations

VI *Histology*

Histologically the utricular nerve section and the saccular macula destruction were properly placed in 18 operated ears One animal out of the originally assigned 10 monkeys showed a severe labyrinthine reaction histologically and therefore was excluded The utricular nerve was cut near totally but in some cases a small neural segment in the region neighboring the lateral ampullary nerve was not cut (Fig 4) The saccular macula destruction was complete in almost all cases The crista ampullaris lateralis was intact except for one ear in which the thickness of sensory epithelium reduced slightly at its summit without any hair cell loss

Fig. 4. Photomicrograph show[illegible] ww of destroyed macu- l[illegible] utricul and otolithic membrane. The crista ampullaris lateralis is intact morphologically. Some precipitate exist. Hematoxylin-eosin stain. ×70

The crista ampullaris superior and the crista ampullaris posterior were intact in all ears.

COMMENTS

It has been reported that, even though the applied rotatory stimulus was the same, the PRN duration became shorter in the order of yaw, pitch and roll (Melvill Jones et al. 1964, Benson & Bodin 1966*a*). *In the case of the* cephalo-caudal rotation, the PRN decay was faster in earth horizontal axis rotation than that in vertical axis rotation (Guedry 1965, Benson & Bodin, 1966*b*). When the cephalo-caudal rotation was applied in horizontal axis, the difference in stopping position had no direct influence to the PRN decay (Benson & Bodin 1966*c*). But, the PRN decay was influenced by the change or adjustment of the posture *immediately* after the cessation of cephalo-caudal rotation in vertical axis. When the head was moved into horizontal position (supine, prone and lateral positions) from the vertical immediately after the cessation of rotation, *SPEV of PRN* declined faster. This *effect* was minimal in the case of pitch or roll *rotation*.

From those rotation studies, it is reasonably speculated that the man's vestibular function could be most efficient in a perpendicular plane to the gravity direction and affected by the change of gravity direction. When the gravity direction is changed, the effect from *simultaneous* existence of change in the proprioceptive cues, or change in the circulatory system, etc. to the nystagmus cannot be *ignored*.

In the present study in sub-human primates

we used cephalo-caudal rotation in upright vertical axis 9° tilt axis and 18° tilt axis The impact of rotatory stimulus cessation might not be precisely equal every time however a small standard error of the mean (of SPEV) indicate the good repeatability The average of maximum SPEV in normal monkeys immediately after the cessation of 200 /sec stimulus (straight vertical rotation) was 165 / sec in this series and was 172 /sec in our previous study (Igarashi et al 1978)

When the rotation axis was tilted off vertically direction of gravity cue continuously changes whereas the intensity of angular acceleration was constant In man this is a very stressful stimulus condition and produces motion-sickness symptoms Similarly in squirrel monkeys prodromal symptoms were observed

The PRN decay looks dependent to the relationship between rotation plane and the gravity direction This is most probably the result of macula-semicircular canal convergence (which is neurophysiologically confirmed in the vestibular nuclei in other animals) and may not be the result from inter-semicircular canal convergence (Correia & Guedry 1966 Markham & Curthoys 1972 Kubo et al 1977) insofar as no difference was found among these different axes after the bilateral macular ablation

In order to evaluate the macula-semicircular canal interaction it is a reasonable approach to study PRN decay in the weightlessness condition However no difference was reported between 1-G and 0-G during the test within a limited time of exposure to 0-G in the parabolic flight (Jackson 1966 Oosterveld & van der Laarse 1966)

As far as the straight vertical axis rotation is concerned our previous study on damped pendular rotation nystagmus (Igarashi et al 1977) showed a reduction of slow phase eye speed after the macular ablation The present study of PRN also showed a similar post-ablative trend Light-microscopically the cristae ampullares were not damaged by the surgery of macular ablation Therefore SPEV decline could be reflectory to some unnatural situation of macula-lateral semicircular canal interaction caused by the elimination of tonic macular inputs or due to decreased excitability in vestibular nuclei after the macular ablation.

After bilateral macular ablation there was no difference in PRN decay curves among three rotatory axes In this case the perception of the gravity direction was done through other receptors than the maculae Even though we consider that the dependency to the remaining gravity receptors must be enhanced due to the necessity of gravity perception, it was found from this study that the macular endorgans have far dominant importance

The effect from two stage ablation to the PRN decay was different between rotations in upright vertical axis 9° tilted axis and 18° tilted axis The decay increase was clearly observed in the upright vertical rotation whereas it was less significant in the 18° tilted axis

ZUSAMMENFASSUNG

Die langsam-phasische Augengeschwindigkeit (SPEV) und die Dauer des Nachdrehnystagmus (PRN) wurden an Totenkopfaffen (*Saimiri sciureus*) nach einer linear beschleunigten Standortsumdrehung (0–200°/sec mit 1°/sec² Winkelbeschleunigung) untersucht Beim Vergleich zwischen der Umdrehung um die senkrechte Achse und der Umdrehung um die um 9° bzw 18° gegenüber der Senkrechten schiefe Achse wurde in den schiefachsigen Umdrehungssituationen schnelleres Abklingen sowohl der SPEV als auch der Dauer festgestellt Der durch die Schiefstellung der Umdrehungsachse hervorgerufene Unterschied der nystagmischen Abklingkurven konnte von der Konvergenz von Eingaben der Maculae und der Bogengänge herrühren Anschließend wurde (zweistufige) zweiseitige maculare Ablation durchgeführt Der Unterschied der Nystagmus-Abklingkurven zwischen drei verschiedenen Umdrehungen war vermindert daher war die Änderung der Gravitationsrichtung, die durch andere Gravitationsrezeptoren als maculare Endsinneszellen wahrgenommen wurde minimal und erzeugte keinen Unterschied in drei verschiedenen Umdrehungen.

ACKNOWLEDGEMENTS

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M. Igarashi
Otological Research Laboratories
Baylor College of Medicine
Houston, Texas 77030, USA

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RELATIONSHIP BETWEEN THE HEAD S AND THE BODY'S CENTER OF GRAVITY DURING NORMAL STANDING

K Taguchi

From the Department of Otolaryngology Faculty of Medicine Shinshu University Matsumoto Japan

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Abstract An apparatus for the recording of head movements has been developed and a basic study was performed to determine the normal values of head movement and to estimate the significance of the head movement as a righting reflex The locus traced by the head closely resembled that of the center of gravity though shorter in overall length The head also moves more slowly than the center of gravity The phase delay of its movement was observed during standing with eyes closed These results suggest that the head's movement is controlled by the body s center of gravity and that the latter moves in order to keep the head steady maintaining the eyes in normal position.

The upright position which is maintained by vision proprioception exteroception and vestibular function has long been considered an indicator of the righting reflex However the mechanism of the righting reflex has not been completely elucidated

The movement of each component of the body results in a synthetic vector for the whole body of the subject The relationship between the movements of two selected points on the body will let us know the characteristics of the righting reflex There are several reports which make a distinction between the center of pressure and the center of gravity especially during human physical activities(Elftman 1938 Murray et al 1967) Measurement of the movement at two points on the body including the body s center of gravity would be useful to explain the body s balance mechanism

The author has developed a new technique to record the movement of the head and recorded simultaneously the movement of the body s center of gravity and the head's movement in order to investigate the characteristics and the mechanism of the righting reflex during normal standing

MATERIALS AND METHODS

1 *Subjects*

A total of 20 healthy subjects between 18 and 30 years of age 10 males and 10 females, took part in the experiment Each subject was placed on a platform beneath a video camera. He was asked to stand with feet together and to look straight ahead at a target 2 m in front

2 *Apparatus*

(a) *Recording the movement of the body s center of gravity* (Fig 1)

The static sensonograph constructed on the basis of the strain gauge technique was used to obtain a continuous record of the movement of the body s center of gravity (CG) from a subject standing on the horizontal platform The output from the sensonograph in terms of voltage was connected to a plotting unit The information obtained was at the same time supplied to a data recorder which separately stored the data of lateral and anteroposterior CG movement on the magnetic tape

(b) *Recording the head movement* (Fig. 2)

The cephalographic system consists of an industrial television camera which picks up the

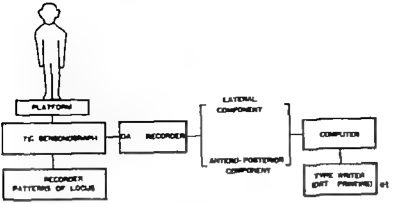

Fig 1 Recording the movement of the body' center of gravity

movement of a light-emitting diode (LED) on the helmet of the standing subject and transmits the movement in terms of voltage to a preamplifier connected to a wave control unit and finally to an X Y recorder The information so obtained can at the same time be fed into the data recorder and be stored on magnetic tape

The recorded data on the tape was fed into digital computer for further analysis

RESULTS

Locus traced by the head movement and the CG The locus of the head movement and that of the CG recorded at the same time in normal subjects are shown in Fig 3 The head locus and the CG locus of the same subject showed a marked similarity except for high density in the CG locus.

Total length of locus traced by the head

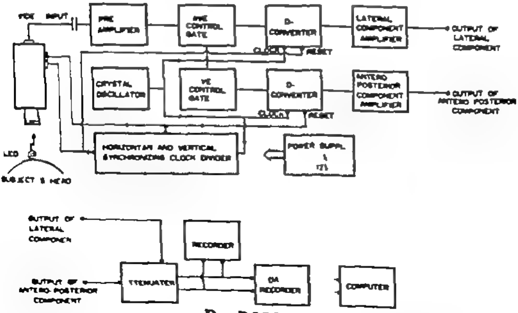

Fig Recording the head movement.

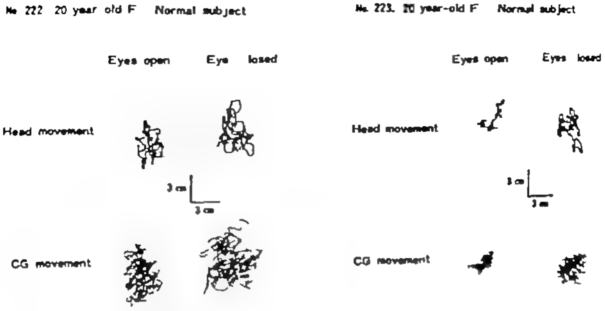

Fig 3 Locus traced by the head movement and the CG recorded simultaneously during 1 minute of standing.

movement and the CG In normal subjects the total lengths of locus traced by the head movement with eyes open and with eyes closed were smaller than those of the CG (Table I) This finding resulted from the difference in the traced locus density

Ratio of total length of locus traced by head movement or CG with eyes closed compared with that with eyes open (EC/EO) EC/EO could be considered to indicate the grade of optical compensation of the righting reflex in the body s stability mechanism The ratio was larger for the CG than for head movement (Table I)

Ratio of the anteroposterior component to the lateral component (Y/X) The ratio (Y/X) denotes the characteristic of the extent of the head movement and the CG movement. The smallest ratio (Y/X) was observed in CG movement with eyes closed The anteroposterior component was relatively larger in the head movement than in the CG (Table I)

Time course of the averaged position of head and CG The time course of the averaged position was calculated each 10 sec and plotted on the co-ordinates The position of the head and the CG showed almost the same time course with eyes open while there was some difference between that with eyes closed showing some delay of the head position (Fig 4)

Frequency spectra of the movement The frequency spectra of the movement were described by using Fourier analysis Generally speaking the frequency spectra of the head's movement showed a dominantly slower frequency than that of the CG movement, and there was a frequency shift to a faster frequency in the head movement and the CG movement when the subject stood with his eyes closed (Fig 5 and Table II)

Table I *Total length EC/EO and Y/X of the head movement and the CG movement*

Means±standard deviations during 1 minute of standing (*N*=20)

	Head movement	CG movement
Total length		
with eyes open	36.8± 8.0	48 6±10.2
with eyes closed	69 7±10.5	76.1±13 1
EC/EO	1 41± 0.39	1.50± 0 44
Y/X		
with eyes open	1 18± 0.21	1 09± 0.21
with eyes closed	1 08± 0.24	0 96± 0.23

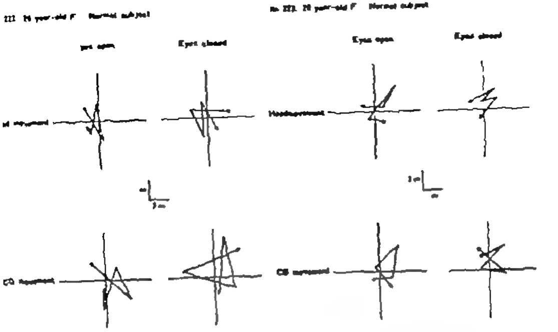

Fig. 4. Time course of the averaged position. The time course of the averaged position in the head and the CG was calculated every 10 sec and plotted on the co-ordinates whose origin was the position of the first 10 sec. Circle denotes the position of the second 10 sec and arrow indicates the position of the sixth 10 sec.

DISCUSSION

Vierordt graphically recorded head movement for the first time in 1862. In 1886 Mitchell & Lewis stated that patients were tested for their relative ability to stand firm by placing them in front of a horizontal scale graduated in inches and located on a level with the ears. Since then many techniques for the recording of head movement have been developed. As examples, the following can be mentioned: 1) Smoked paper and an index (Hindale 1887, Dana 1897). 2) Silk thread, pulley and kymograph (Hindale 1890). 3) Cephalograph which records graphically the head movement in two dimensions—in the sagittal and in the frontal plane (Knauer & Maloney 1914). 4) Ataxiameter to record the head movement by using adders to turn freely in one direction (Miles, 1922; Ewald 1942). 5) Accelerometer (Egmond et al. 195 , Kitahara, 1965). 6) Transducer and polygraph (Tokita et al. 1970). 7) Optical method using a camera (Goldberg 1943, Orma 1957, Jarrige 1968, Claussen 1970) or using an industrial television camera (Kapteyn & De Wit, 1972, Kawano & Tokumasu 1972).

In addition to the above-mentioned techniques there is some promising research in progress (all effect technique by Uemura et al. 1976; Matrix plate of phototransistors by Kodaira, 1978).

The purpose of recording head movement

Table II. *Averaged divisional frequencies* (1/60~1 Hz)

Mean ± standard deviation during 1 minute of standing (N = 20)

	Head movement	CG movement
With eyes open		
Lateral component	0.39+0.05	0.44±0.05
Anteroposterior component	0.34+0.04	0.42 0.06
With eyes closed		
Lateral component	0.41±0.06	0.49±0.08
Anteroposterior component	0.38±0.06	0.46±0.08

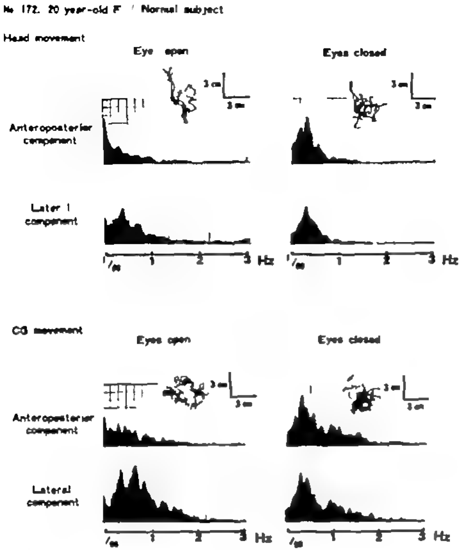

Fig. 5. Frequency spectra of the movement.

has been to find a pathognomonic characteristic (Shimazono 1908, Tokita et al. 1970) or to estimate the head movement itself (Knauer & Maloney 1914). No trial has been made to clarify the significance of head movement in the righting reflex. The author intended to study head movement in relation to the CG in the righting reflex.

According to Smith's model (1957) the human body moves around the ankle axis and the head seems to show maximum sway during standing. The human body is composed from above downwards of several segments (head, trunk, leg and foot) which are not rigidly fixed to each other. Each segment possesses its own center of gravity which must be considered in relation to the point of support on the segment just below and to the area of support on the ground. The maximum of stability and the minimum of constraint would be obtained if the various points of bearing were all in the same vertical line, and if that line fell immediately in the center of the area of support (Miles 1922). Therefore the human body does not move as a rigid stick, but different portions of the body sway in different manners.

The head's movement in relation to the CG constitutes the mechanism of the righting reflex. The head, containing the brain, the eyes and the vestibular organs, must maintain its balance so as to fix the gaze upon an object. In this way one orientates one's position in space. In order to attain these functions the CG seems to move appropriately.

ZUSAMMENFASSUNG

Ein Aufnahmeapparat für die Kopfbewegung ist entwickelt und ein Studium wurde durchgeführt, um den normalen Wert der Kopfbewegung zu erhalten und die Bedeutung der Kopfbewegung als einen Stellreflex zu untersuchen. Der vom Kopf gezeichnete Locus ähnelt sehr dem Schwerkraftzentrum des Körpers. Aber die totale Länge des durch den Kopf gezeichneten Locus war kürzer als die des Schwerkraftzentrums. Der Kopf bewegt sich langsamer als das Schwerkraftzentrum, und die Phasenverzögerung der Kopfbewegung wurde beobachtet während Stehen mit geschlossenen Augen. Diese Ergebnisse lassen ermuten, daß die Kopfbewegung durch das Schwerkraftzentrum des Korpers kontrolliert wird und daß das Schwerkraftzentrum den Kopf aufrecht hält und somit die Augen ihre normale Position behalten.

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K. Taguchi, M.D.
Department of Otolaryngology
Faculty of Medicine
Shinshu University
3-1-1 Asahi
Matsumoto
Japan

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EMBRYOLOGIC DEVELOPMENT IN VIVO AND IN VITRO OF THE DARK CELL REGION OF THE MAMMALIAN CRISTA AMPULLARIS

Matti Anniko

From the Department of Oto-rhino-laryngology Karolinska Sjukhuset and King Gustaf V Research Institute Karolinska Institutet Stockholm Sweden

(Received August 7 1979)

Abstract The area comprising the dark cells around vestibular organs was analysed with regard to embryologic development and maturation from the 13th gestational day (otocyst stage) (CBA/CBA mouse) to birth at the 21st gestational day when the organ reveals a morphologic maturation of inner ear gross structure and as also cytodifferentiation of vestibular hair cells. The development occurred parallel in vivo and in vitro Electron-optically dense pigments below the dark cell region were first identified on the 15th gestational day On the 16th gestational day the future dark cells have their mitochondria accumulated to the infranuclear region of the cell From the 17th day onward intercellular spaces develop close to the basal membrane forming at birth a meshwork of interdigitations Prior to birth the future dark cells reveal an increase in electron density after staining. At birth the morphological configuration of the dark cell area appeared quite mature

Developmental studies on the inner ear have in general focused their interest on the differentiation of hair cells (Van De Water & Ruben 1971 Anniko et al 1979) During the prenatal period the embryogenesis of the labyrinth has been described as occurring in parallel in vivo and in vitro

A considerable difference in the time relationship occurs in structural maturation between the vestibular organs and the cochlea the latter being rather immature at birth in the CBA/CBA mouse While the cochlear hair cells are present in all coils at this juncture the cells comprising the stria vascularis pass their terminal mitosis post partum (Ruben 1967) In contrast Anniko et al (1979) reported that the crista ampullaris has a rather mature configuration at birth with regard to gross morphology The hair cells too are structurally mature while their innervation is not completed until 3–6 days post partum (Nordemar et al 1979 to be published)

The present study describes the embryologic development in vivo and in vitro of the dark cell region of the crista ampullaris in the CBA/CBA mouse In the literature these cells are regarded as the site of vestibular endolymph production

MATERIAL

In vivo The total material consisted of 24 embryos and 3 newborn animals The inner ears of 3 embryos were taken for morphological analysis each day during development starting at the 13th gestational day

In vitro Inner ears were explanted on the 13th and the 16th gestational day respectively and followed in vitro until the time corresponding to birth As in the in vivo group of specimens the results are based on the study of 6 labyrinths at each stage/day of embryologic development

METHODS

Organ culture technique This has been described in detail by Anniko et al (1978 1979) based on the work of Van De Water (1976)

Supported by grants from Karolinska Institutet, The Swedish Society of Medical Sciences Tysta Skolan and The Swedish Medical Research Council (grant no 12X 7?0)

Fig. 1. Light microscopy (LM). Fixation of the 16th gestational day inner ear in 4% formaldehyde. A thick cartilagenous capsule (*asterisks*) surround the membranous inner ear structures at this stage. *CA* crista ampullaris; *U* utricle; *S* saccule. The area comprising the dark cells is indicated by arrows.

Most organ cultures of the developing otocyst have been photodocumented daily.

Morphologic processing

I. *The main part of the material.* During the early stages of embryologic development (days 13–16) the otocyst was fixed *in toto* with 3% glutaraldehyde in 0.133 M sodium phosphate buffer. Later (days 16–21) the inner ear was divided into vestibular and cochlear portions to facilitate a better penetration of the fixative into the membranous labyrinth. On the 16th gestational day the inner ear is enclosed within a cartilaginous capsule which impairs the rate of fixation.

After a rinse in buffer the specimens were postfixed in 1–2% osmic tetroxide in Veronal acetate buffer (pH 7.2) and dehydrated in increasing concentrations of alcohol. Embedding was performed in Epon mixture. Light microscopic sections were stained with toluidine blue. Based on the light microscopic findings a part of the material was sectioned for electron microscopy (Philips 400) using uranyl acetate and lead citrate for staining.

II. *A smaller part of the total material* was fixed in a solution of 4% formaldehyde and embedded in paraffin. These specimens of the whole inner ear were serial-sectioned and stained with haematoxylin-eosin (Fig. 1).

RESULTS

General considerations

The organogenesis of the vestibular organs was followed during in vitro development (Fig. 2A and B). The pigmentation of the dark cell region was easily identified in the inner ear explants. Concerning the 16th gestational day explants, no changes in gross morphology were observed in the vestibular region during the in vitro culture (Fig. 3).

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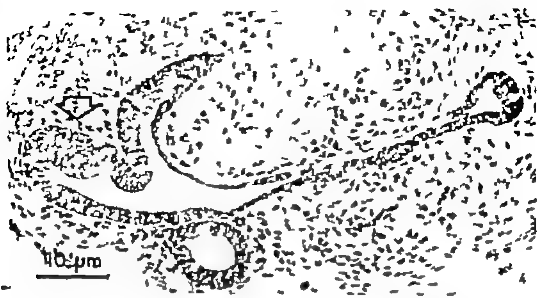
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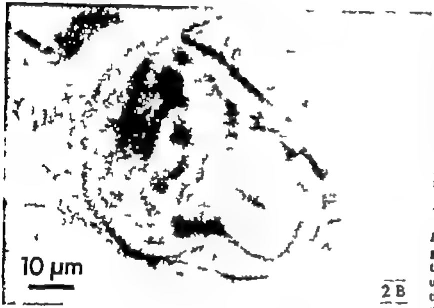

2 B

Fig 2 LM of live inner ear explant (A) 13th gestational day inner ear anlage immediately after explantation. (B) Inner ear explanted on the 13th gestational day and cultured in vitro for 2 days.

Fig 3 LM of live 16th gestational day inner ear anlage Morphogenesis is almost completed Pigments in the vestibular dark cell region are visible

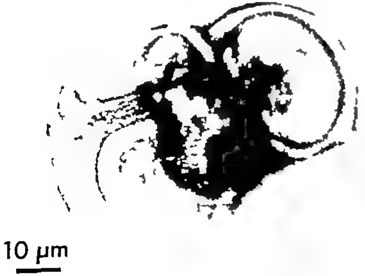

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Fig 4 LM Section through the late 13th gestational day inner ear anlage. Folding of the otocyst has started to form the vestibular part of the labyrinth Proliferation of the epithelium has started (*arrow*) to form the vestibular organs. Type of future cells cannot be distinguished at this stage of development

Fig 5 LM Serial sectioned 16th gestational day inner ear The configuration of supporting tissue in the crista ampullaris (*CA*) and the utricle (*U*) is still immature with several layers of cell nuclei. A hair cell layer is clearly visible The region of future dark cells (*arrow*) contains 1 2 layers of nuclei but is distinctly separated from surrounding structures.

Fig 6 LM 13th gestational day inner ear explant cultured in vitro for 3 days. In this particular specimen the crista ampullaris (*CA*) and the utricle (*U*) are still not separated *in toto* On the opposite side of the crista ampullaris (the cupula is indicated by arrows) a region is observed which might become the elongated cells of the planum semilunatum. The dark cell region would therefore be located lateral to these cells.

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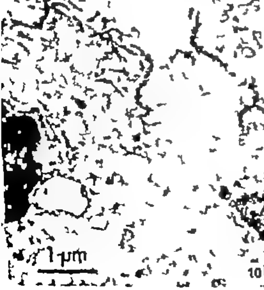

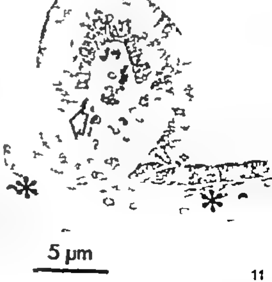

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Sectioned specimens

The 13th gestational day otocyst is still cystic with an epithelium of 1–2 cell layers Late during this stage a folding into the complex vestibular system is indicated (Fig 4) It has to be noted that during organogenesis of the crista ampullaris a proliferation of cells occurs in the hair cell bearing region with its underlying stroma while the area of future dark cells is devoid of such a process

Morphologically no major differences in differentiation of this region were noted between the in vivo and the in vitro group of specimens either if explanted on the 13th or 16th gestational day and followed to the point in time corresponding to birth Electron optically dense pigments were identified in vivo on the 15th and 16th gestational days which however was extremely rare in in vitro explants of the 13th gestational day inner ear even if cultured until partus (cf Fig 3)

Sections through the 16th day inner ear often reveal a mature relationship between the gross morphology of the crista ampullaris and the adjacent secretory region of dark cells (Fig 5) but in some instances more frequently in in vitro specimens the region between the crista ampullaris and the utricle was minimal separating the two organs only by a few cells (Fig 6) This is generally overcome however by increasing the culture time

The cells in the dark cell area possess no specific morphology such as hair cells have in early development (Fig 7) On the 16th gestational day mitochondria are often accumulated in the infranuclear part of the dark cell close to the basal membrane Short clumsy kinocilia on the dark cells can be observed even immediately prior to birth From the 17th gestational day onward (in vivo 13+4 and 16+1 days in vitro respectively) intercellular spaces develop close to the basal membrane whereafter this process continues upwards between the cells (Fig 8) On the 20th day the dark cells often reveal a meshwork of interdigitations with a large number of mitochondria In this region (Fig 9) During this period the cells

Fig 7 Electron microscopy (*EM*) Late 14th gestational day inner ear Some cells facing the otocyst lumen have their microvilli regularly arranged on their surfaces as compared with surrounding cells. Furthermore their cytoplasm is not stained so intensely These characteristics are indicative of their future development into hair cells Rootlets from microvilli are observed to penetrate into the cell however without the formation of a cuticle. It should be noted that the microvili on the surface of the (probably future) hair cell at this stage are no thicker than those of surrounding (probably supporting) cells.

Fig 8 EM 18th gestational day inner ear Cells of the dark cell region The cells contain numerous mitochondria, both smooth and rough endoplasmic reticulum as well as a large number of free ribosomes. Folding of the plasma membrane is starting in the direction of the basal membrane (*BM*) causing intercellular spaces. The cell cytoplasm shows increased electron density

Fig 9 EM 16th gestational day inner ear explant cultured in vitro for 4 days. The basal part of dark cells now has a complex system of interdigitations. The cytoplasm contains numerous mitochondria and groups of free ribosomes *BM* basal membrane (*arrows*)

Fig 10 EM 13th gestational day inner ear/otocyst cultured to a time corresponding to partus i.e 8 days in vitro The dark cells have a mature configuration with regard to electron density and microvilli on the cell surface towards the endolymphatic space Many small vesicles are present in the cells.

Fig 11 LM Crista ampullari of the newborn CBA/CBA mouse 1–2 rows of supporting cells below the hair cell layer The elongated transitory cells are now identified (*arrow*). The region of dark cells is indicated by asterisks.

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STUDIES OF THE VESTIBULO-OCULAR REFLEX AND VISUAL–VESTIBULAR INTERACTIONS DURING ACTIVE HEAD MOVEMENTS

Masahiro Takahashi, Takuya Uemura and Takehisa Fujishiro

From the Department of Otolaryngology, Tokyo Women's Medical College, Tokyo, Japan

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Abstract. The ratio of slow phase eye speed to head speed (Gain) during voluntary sinusoidal head rotations at 0.33, 0.67 and 1.0 Hz was studied in normal subjects under various target presentations. With mental arithmetic in the dark, the mean value of the Gain was 0.8 irrespective of turning frequencies. When subjects looked at or imagined (in the dark) a spatially fixed target, the Gain was always maintained at unity. The Gain measured with head-fixed target was 0.[illegible], 0.33 and 0.54 at 0.33, 0.67 and 1.0 Hz, respectively. However, imagining a target in this condition failed to suppress the Gain obtained with mental arithmetic in the dark. When a target moved twice as fast as the head (pursuit of the reflected image in a head-fixed mirror), the Gain was 0.60, 0.11 and 0.30 at 0.33, 0.67 and 1.0 Hz, respectively.

Although we experience active head rotations of faster than 100°/sec daily without any deterioration of visual acuity, the maximum pursuit speed of the retino-oculomotor reflex is 60–70°/sec at most, as shown in the adaptation limit of optokinetic nystagmus (Mackensen, 1954; Honrubia et al., 1968; Miyoshi & Pfaltz, 1974; Takahashi et al., 1978). Regarding the vestibulo-ocular reflex, the ratio of slow phase eye speed to head speed in the dark (the gain of the vestibulo-ocular reflex, VOR Gain) has been measured in various mammals and is reported to be less than unity in the case of passive rotation (man: Meiry, 1971; monkey: Skavenski & Robinson, 1973; cat: Robinson, 1976; rabbit: Baarsma & Collewijn, 1974).

Thus, it must be concluded that neither of the retino-oculomotor and vestibulo-ocular reflexes can accomplish visual fixation during quick head movements. However, Barr et al. (1976) showed that the VOR Gain is maintained at a level very close to unity by making subjects try to keep their eyes on an imaginary target fixed in space.

Although the nature of the interaction between the vestibulo-ocular and retino-oculomotor reflexes has been already investigated, most of the experiments involved passive rotations, and the nature of vestibulo-visual interaction during active head rotations, which seem most natural, is still unknown. In this paper, we observed the input–output relationships (target speed – eye speed) in identical subjects under various conditions in which visual and vestibular stimulations were changed from cooperative to uncooperative.

METHODS

In this study, rotatory stimulation was given by active horizontal sinusoidal head turnings. Eye movements were recorded by d.c. ENG through bitemporal leads on the outer canthi. Head rotation was recorded by attaching a head displacement angle detector, a cross of two small rectangular pieces of alloy sealed in a low column case working as a terrestrial magnetic sensor, to the head. By using this equipment, free head rotations on a horizontal plane can be measured up to 50° right and left from the midpoint with 0.5° accuracy (Uemura et al., 1976).

The rotation speed was controlled indirectly by regulating the frequency and amplitude of sinusoidal rotations. For regulation of the frequency, we instructed the subjects to turn their heads alternately and smoothly to the

Anniko M & Wroblewski R. 1979*a* Elemental composition of the mature inner ear *Acta Otolarj ngol* (Stockh) in press

Anniko M & Wroblewski R. 1979*b* Elemental composition of the developing inner ear To be published

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Matti Anniko M.D
Department of Oto-rhino-laryngology
Karolinska Sjukhuset
S 104 01 Stockholm
Sweden

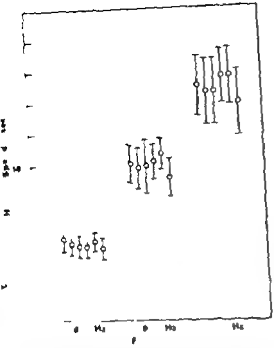

Fig. 2. Mean and S.D. of maximum head turning speed at different frequencies, from left to right: alert in dark, "target fixed in space", "imaginary target in space", "head-fixed target", "imaginary head-fixed target" and "target in the mirror".

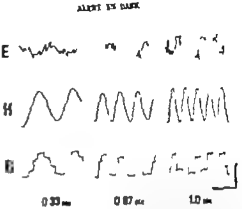

Fig. 3. Recordings with mental arithmetic in the dark. Figs. 3–6 show recordings of the same subject in different conditions. From top to bottom, eye movements (E), head movements (H) and gaze movements (G) are shown. Vertical and horizontal lines at lower right represent 40° and 2 sec, respectively (also in Figs. 4–6).

brated whenever the testing conditions were changed.

The head and eye speeds were ascertained by measuring the tangent angles of the recorded waves in which the frequency and amplitude of the head rotations were most stable. The subjects were 20 healthy adults aged 19 to 35 (10 males and 10 females, mean age 28.5) who had no abnormal neuro-otological sign. The tests were given once a week to avoid training effects, and repeated three times to determine reliability.

The maximum head speed (mean and S.D.) measured in the three repeated trials of the 20 subjects in the six different testing conditions are shown in Fig. 2. Mean head turning speeds were 50, 100 and 150°/sec at 0.33, 0.67 and 1.0 Hz respectively, although in target in the mirror the head speeds were slightly slower than in the other testing conditions.

RESULTS

1. Mental arithmetic in the dark (alert in dark)

Although the ENG recordings showed manifest nystagmus beating in the same direction as the head rotation, gaze movements, i.e. summation of the eye and head movements, rarely occurred except during the quick phase of nystagmus (Fig. 3). The ratio of the maximum slow phase eye speed to the corresponding head turning speed (VOR Gain) was 0.8 on average and it was not affected by differences in the turning frequency (Table I, Fig. 7). The analysis of variance showed no significant difference among the VOR Gains at different frequencies ($P>0.05$).

The mean value of the VOR Gain obtained from nine trials (three repeated trials in three different frequencies) ranged widely among the subjects, from 0.63 to 1.05. We examined the correlation coefficient between each pair combination of the three repeated trials in order to ascertain the reproduction of the variation in the VOR Gain among the subjects. Significant correlation was noted at 0.67 Hz ($0.01<P<0.05$) but not at 0.33 and 1.0 Hz, as shown in Table II.

To summarize these findings, the VOR Gain

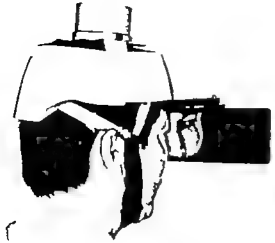

Fig 1 Head rotation angle detector with a mirror installed diagonally in front of the eyes for reversing the visual stimulus direction

right and left in time with an electric metronome Head rotations were repeated at 0 33 0 67 and 1 0 Hz The amplitude of the sinusoidal rotation was 40° We continually checked and regulated the frequency and amplitude of the head rotations during the tests

For visual regulation of the eye movements we used two kinds of target—a small red lamp on the wall and a small red lamp on the tip of the rod (30 cm in front of the eyes) connected to the helmet In addition a light mirror was placed diagonally in front of the eyes for reversing the direction of the visual target (the lamp on the wall) during head rotations (Fig 1) When the subjects rotated their heads with the mirror the reflected image of the lamp on the wall moved in the same direction and at the same speed as the head rotation

With these instruments we observed eye movements head movements and gaze movements (summation of the eye and head movements) under the following visual conditions During the tests the room was kept dark except for the red lamp for visual fixation

1) Mental arithmetic in the dark (alert in dark)
2) Visual fixation on the lamp on the wall
   (*a*) Lamp switched on (target fixed in space)
   (*b*) Keeping the gaze on an imaginary target after the lamp is turned off (imaginary target in space)
3) Visual fixation on the lamp moving with the head
   (*a*) Lamp switched on (head-fixed target
   (*b*) Keeping the gaze on an imaginary target after the lamp is turned off (imaginary head fixed target)
4) Pursuit of the reflected image of the lamp on the wall in the mirror (target in the mirror)

In alert in dark observation of vestibulo-ocular reflex (VOR) during active head rotations is possible The ratio of the visual stimulus speed to head turning speed is 1 0, and −1 0 in target fixed in space head-fixed target and target in the mirror respectively Target fixed in space in which vestibular and visual stimuli drive the eyes in the same direction is regarded as a cooperative condition while head fixed target and target in the mirror in which the stimuli work to inhibit each other may be regarded as uncooperative conditions We can observe the effects of imagining a target on the vestibulo-ocular reflex in imaginary target in space and imaginary head fixed target

The testing procedures were as follows After the helmet with the rotation angle detector and the electrodes for ENG recordings were attached to the subjects heads and outer canthi respectively the subjects were adapted in the dark for 10 minutes Eye movements were calibrated by small red lamps spaced at 20° intervals (visual angle) on the wall Then the subjects were instructed to remember the rotation angle (right and left 40° visual angle) by turning their heads and to rotate their heads smoothly and rhythmically in time to a metronome After getting used to this procedure the subjects were tested successively in alert in dark through target in the mirror The eye movements were cali-

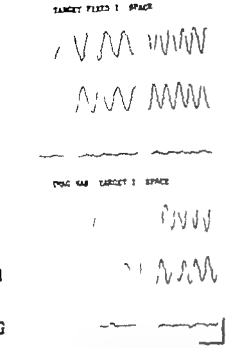

Fig. 4. Recordings with visual fixation on a lamp on the wall. It was hard to find any difference between the recordings in the light (upper traces) and in the dark (lower traces). The eyes moved in the exact opposite direction to the head and the gaze was maintained almost fixed in space.

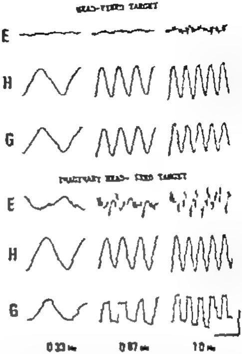

Fig. 5. Recordings with visual fixation on a lamp moving with the head. Even at 0.33 Hz in the light (upper traces), minute nystagmus was observed. The recordings at 0.67 and 1.0 Hz in the dark (lower traces) were almost similar to those with mental arithmetic in the dark (Fig. 3).

3. Visual fixation on a lamp moving with the head (head fixed target and imaginary head fixed target)

If the subjects could accurately fix their gaze on a target in these conditions, eye movements should not be observed at all and the gaze movement should correspond with that of the head. Although most of the subjects could partially maintain their gaze on a lamp during rotation at 0.33 Hz, the fixation became worse at higher frequencies as the vestibular nystagmus became stronger (Fig. 5). The Gain changed remarkably from 0.22 to 0.54 on average according to increase of the turning frequency (Table I, Fig. 7). Changes of the Gain according to changes in the frequency were proved to be highly significant ($P<0.01$). Good reproduction of individual Gains in this condition was certified by the high correlation found between the three repeated trials at identical frequencies (mean correlation coefficient was 0.617, being significant when tested by 1% probability) (Table II).

When the subjects tried to fix their gaze on an imaginary lamp in front of their eyes after the lamp was turned off, the mean Gains were found to be 0.60, 0.75 and 0.78 at 0.33, 0.67 and 1.0 Hz, respectively (Table I, Fig. 7). We found significant difference between the Gains at 0.33 and 0.67 Hz ($P<0.01$), but not between those at 0.67 and 1.0 Hz ($P>0.05$). The reproduction of the Gain at identical frequencies in the repeated trials was worse in the dark than in the light (Table II).

Lack of a visible target is common in both imaginary head-fixed target and alert in dark. When the Gains of these two conditions were compared, they differed significantly at

Table I *Mean and S D of the ratio of maximum slow phase eye speed to corresponding head turning speed (Gain)*

Condition	0.33 Hz	0 60 Hz	1 0 Hz
Alert in dark	0 80±0 10	0.82±0 13	0 79±0 12
Target fixed in space	0 99±0 03	0 99±0 02	1 00±0.05
Imaginary target in space	0 98±0 04	0 97±0 05	0.98±0 06
Head-fixed target	0.22±0.12	0.33±0 14	0.54±0 17
Imaginary head-fixed target	0 60±0 16	0 75±0 16	0 78±0 16
Target in the mirror	−0 60±0.20	−0 11±0 23	0.38±0..3

measured with mental arithmethic in the dark was not manifestly affected by changes of the turning frequency but there was considerable variation in Gain measurements inter individually and intra individually

2 Visual fixation on a lamp on the wall (target fixed in space and imaginary target in space)

Inasmuch as the eyeballs moved in the direction opposite to the head the gaze was well maintained stationary in space (Fig 4) The ratio of the maximum slow phase eye speed to the corresponding head turning speed (Gain) proved to be very close to unity in all subjects regardless of different turning frequencies (Table I Fig 7) Statistically no significant difference was found among the Gains at 0 33 0 67 and 1 0 Hz ($P>$0 05)

The mean value of the nine trials (three repeated trials at different frequencies) varied from 0 95 to 1 03 among the subjects The correlation coefficient among the repeated trials was very small since the Gain varied little among the subjects (Table II)

Even when the subjects rotated their heads while trying to fix their gaze on an imaginary target after the lamp was turned off (imaginary target in space) the gaze was well maintained stationary in space (Fig 4) As in target fixed in space the value of the Gain was very close to unity (Table I Fig 7) There was no significant difference among the Gains at 0.33, 0 67 and 1 0 Hz ($P>$0 05) The individual Gains averaged from the nine trials ranged from 0 90 to 1 07 which was slightly more variable than that found in the light

We found no significant difference among the Gains measured in the light and in the dark at all frequencies ($P>$0 05) Regarding the variance of the Gain significant difference was found between both conditions at 0 67 Hz ($P<$0 025) but not at 0 33 and 1 0 Hz.

From these findings it was concluded that the gaze is well maintained on a spatially fixed target even if the target is imaginary

Table II *Mean value of the correlation coefficient among the Gains in each pair combination out of three repeated trials at identical frequencies*

Condition	0.33 Hz	0 67 Hz	1 0 Hz	Average
Alert in dark	0 .56	0 553	0 438	0 416
Target fixed in space	−0 073	−0 028	0.219	0 039
Imaginary target in space	−0 019	0 088	0 193	0 087
Head fixed target	0 662	0 604	0.584	0 617*
Imaginary head fixed target	0 421	0.467*	0 491	0 460*
Target in the mirror	0 656	0 61	0 701	0 656

and mean significant correlation tested by 5 % and 1 % probability respectively

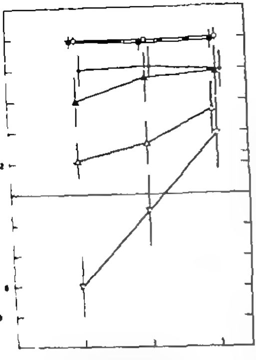

Fig 7 The ratio (mean and S D) of maximum slow phase eye speed to maximum head speed (Gain) in various conditions alert in dark ( ) "target fixed in space" (O) imaginary target in space (●) "head-fixed target" (Δ), imaginary head-fixed target (▲) and "target in the mirror" (?)

suit of a reflected image in the mirror the vestibulo-ocular reflex inhibits performance of the visual function these two conditions may be regarded as an antagonistic relationship between the vestibular and visual stimulations As the Gains observed in these conditions had in fact a highly mutual correlation (Table III) the findings in the case of visual fixation on a target moving with the head might reflect the visual pursuing ability of a target in the mirror The Gain measured in mental arithmetic in the dark showed no significant correlation with the pursuing function in the mirror

DISCUSSION

In these experiments we adopted voluntary head rotations in time to a metronome as rotatory stimulations Observation of eye movements during active head rotations is more appropriate than that during passive rotations when investigating the physiological function of the vestibulo-ocular reflex

It is well known that the strength of the vestibulo-ocular reflex in the dark can be remarkably affected by mental alertness, repetition of stimulation individual differences etc. The active head rotation with mental arithmetic in the dark adopted in the present study might be the most alert state of consciousness in the dark that we can attain We could not find a manifest decrease of vestibular nystagmus (response decline) more or less unavoidable in passive rotations during repeated rotations in the present study

Although the VOR Gain varied not only interindividually (0 63–1 05) but intraindividually even in this alert state the mean value of the VOR Gain (0.8) was not affected by change of the rotational frequency According to Skavenski & Robinson (1973) and Keller (1978) the value of the Gain was 0.85 and 0 92, respectively at 1 0 Hz, in monkeys while according to Meiry (1971) it was 0 65 in humans As the VOR Gain varied among different individuals and at repeated trials even in the same subjects in the most conscious state we have to conclude that the value of the VOR Gain obtained in the dark does not reflect the true functional state of the vestibulo-ocular reflex

On the other hand when the subjects imagined a stationary point in space in the dark the gaze was spatially fixed without exception We could not find any significant difference in the average and variation of the Gains obtained in the dark and those in the light Barr et al (1976) reported that the VOR Gain in the dark is maintained at unity in passive rotations while imagining a spatially stationary point on the wall From these results it is concluded that the VOR Gain can be maintained at unity through the central modification of vestibular inputs even without visual or cervical proprioceptive information.

Table III *In this table the correlation coefficient was tested between the Gains in some pair combination of different conditions*

Condition	0.33 Hz	0 67 Hz	1 0 Hz	Average
Alert in dark & Head-fixed target	0.290	0.260	0 434	0 328
Alert in dark & Imaginary head-fixed target	0 448*	*0 444*	*0.515*	0 469*
Alert in dark & Target in the mirror	−0.219	−0 088	−0.213	−0 173
Head-fixed target & Imaginary head-fixed target	0 512	0.354	0 643	0.503
Head-fixed target & Target in the mirror	−0.859*	−0 743**	−0 657	−0 753**

* and ** mean significant correlation tested by 5% and 1% probability respectively Italics represent combinations in which analysis of variance showed no significant difference tested by 5% probability

0 33 Hz ($P<0$ 01) but not so at 0 67 and 1 0 Hz ($P>0$ 05) Significant correlation was found between the Gains in both conditions (Table III) On the other hand we could not find any correlation between the Gain measured with gaze fixation on a target moving with the head in the light and that with mental arithmetic in the dark

It was proved in this segment of the experiments that a visible target is an essential factor for maintaining the gaze on a target moving with the head and that even in the light gaze fixation is poor and strongly affected by the speed of the head rotation Although the Gain measured with mental arithmetic in the dark was slightly suppressed by imagining a target moving with the head at 0 33 Hz, it was considerably less than that accomplished by visual suppression

4 Pursuit of the reflected image of a lamp on the wall in a mirror (target in the mirror)

In this condition the target movement in the mirror corresponds with the head movement Accordingly if the visual pursuit was successful the eye movement in the orbit coincide with the head movment and the amplitude of the gaze movement should be twice as large as the head movement (Gain = −1 0) In actual observation the subjects could not pursue a target very well even at 0 33 Hz because of the superimposed vestibular nystagmus (Fig. 6) As a result the eye movements became irregular

The mean Gains were −0 60 −0 11 and 0 38 at 0 33 0 67 and 1 0 Hz, respectively (Table I Fig 7) Even in the slow rotation at 0 33 Hz the average eye pursuing speed attained was only 60% of the target speed in the mirror However the reproduction of individual Gain in the repeated trials was excellent (Table II) and the correlation coefficient averaged at all frequencies ($R=0$ 656) showed the highest value among the six testing conditions

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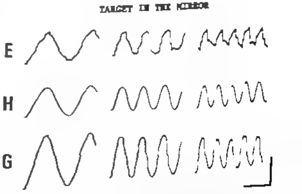
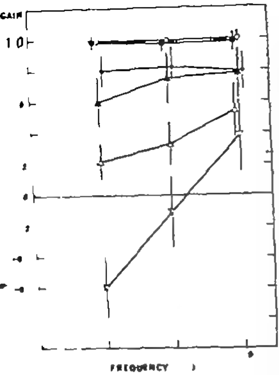


Fig 6 Recordings with a target in the mirror According to the increase of turning frequency the pursuit became remarkably worse and irregular eye movements were involved



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Head-fixed target & Imaginary head-fixed target	0 51	0.354	0 643	0 503*
Head-fixed target & Target in the mirror	−0 859*	−0 743	−0 657*	−0 753**

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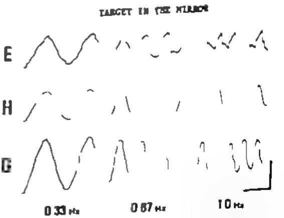

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found in imaginary target in space in this study. Thus, it was suspected that even if the subjects are given contradictory visual and vestibular stimulations, the VOR Gain should always be maintained at unity by the vestibulo-ocular reflex during active head rotations when the modulation of the Gain responsible for the pursuit eye movements is discounted.

CONCLUSION

In order to study the role of the vestibulo-ocular reflex for visual fixation and pursuit during voluntary head rotations, we devised a new test in which subjects rotate their heads *sinusoidally* over 40° in time to a metronome (0.33, 0.67 and 1.0 Hz) under the following visual conditions:

1) Mental arithmetic in the dark. 2) Visual fixation on a lamp on the wall. 3) Visual fixation on a lamp moving with the head. (2 and 3: a) in the light, b) in the dark (imagining a lamp)). 4) Visual pursuit of the reflected image of the lamp on the wall through a mirror placed diagonally in front of the eyes.

We tested 20 normal adults and obtained the following results:

1. In the trial with mental arithmetic in the dark, the mean value of the ratio of slow phase eye speed to head speed (Gain) was about 0.8 irrespective of head turning frequencies. The Gain varied even in the same subjects (S.D. was about 10% of the mean value) on repeated trials as well as among different subjects (0.63–1.05).

2. The Gain measured during visual fixation of a lamp on the wall was always maintained at a level very close to unity, not only in the light but in the dark as well.

3. The Gain measured with visual fixation on a lamp moving with the head increased with turning frequencies (0.22, 0.33 and 0.54 at 0.33, 0.67 and 1.0 Hz, respectively). Imagining a lamp failed to suppress the Gain measured with mental arithmetic in the dark (0.60, 0.75 and 0.78 at 0.33, 0.67 and 1.0 Hz, respectively).

4. When the mirror was used, the reflected image of the lamp moved spatially in the same direction and twice as fast as the head did. The Gain was −0.60, −0.11 and 0.38 at 0.33, 0.67 and 1.0 Hz, respectively. Although the individual's pursuing ability did not correlate with the Gains measured with mental arithmetic in the dark, they proved to have a highly significant correlation with the Gains under visual fixation on the target moving with the head.

ZUSAMMENFASSUNG

Wir untersuchten das Verhältnis der Langsamphasenaugengeschwindigkeit zur Kopfgeschwindigkeit (Verstärkungsfaktor) während freiwilliger sinusoidalen Kopfrotationen bei 0,33, 0,67 respektiv 1,0 Hz unter Vorgabe verschiedener Ziele. Mit Kopfrechnen im Dunkeln war der Wert des Verstärkungsfaktors durchschnittlich 0,8 unabhängig von der Rotationsfrequenz. Schaute die Versuchsperson auf ein wirkliches oder (im Dunkeln) vorgestelltes im Raum fixiertes Ziel, blieb der Wert einheitlich. Der mit kopffixiertem Ziel gemessene Wert war 0,22, 0,33 respektiv 0,54 bei 0,33, 0,67 respektiv 1,0 Hz. Hingegen konnte das Vorstellen eines Zieles in diesem Zustand den mit Kopfrechnen im Dunkeln erhaltenen Wert nicht unterdrücken. Bewegte sich ein Ziel mit doppelter Geschwindigkeit des Kopfes (Verfolgung des Spiegelbildes in einem kopffixierten Spiegel), war der Wert −0,60, −0,11 respektiv 0,38 bei 0,33, 0,67 respektive 1,0 Hz.

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The fact that even in the rabbit whose retina lacks the fovea the gaze is fixed in space except for the time of saccades during free head rotations (Collewijn 1977) supports the contention that visual fixation during head rotations is executed by the active vestibulo-ocular reflex (Robinson 1974)

Visual fixation was incomplete even at 0 33 Hz (maximum head speed was about 50 /sec) when a target moved with the head Although this result represents failure of visual fixation due to the vestibulo-ocular reflex it may be considered that the vestibulo-ocular reflex is suppressed by the vision The vestibularly induced nystagmus became noticeably dominant according to the increase of turning frequency (increase of head speed)

As the Gain is theoretically null when the head is stationary the line connecting values of the Gain at 0 33 0 67 and 1 0 Hz in addition to 0 Hz could be regarded as being almost straight Thus it seems likely that the suppression of the Gain when a target moves with the head decreases in proportion to the increase of the turning frequency If the Gains are compared among different testing conditions it is proved that the Gain found with an imaginary target is close to that found with a visible target when the target is on the wall and close to that found in mental arithmetic in the dark when the target is moving with the head This finding suggests that the vestibulo-ocular system is equipped with a mechanism that immediately adjusts the Gain to unity but not with a mechanism that suppresses the strength of the vestibulo-ocular reflex In other words as the vestibulo-ocular reflex functions to keep the gaze stationary irrespective of visual conditions visual fixation on a target moving in space depends exclusively on the retino-oculomotor reflex

Although visual pursuit of a reflected image through a mirror seems to be a very unnatural stimulus condition insofar as the vestibular and visual stimuli move in the opposite directions in our daily lives such as when we turn our heads to look at a vehicle passing at high speed in front of the eyes the relationship of vestibular and visual stimulations is very similar

The Gain should be more variable in this condition than in any other because it can change from −1 0 to 1 0 As the vestibular nystagmus became stronger according to an increase in the turning frequency the pursuing eye movements were superimposed with frequent saccades to make up for the poor pursuits and the vestibularly induced nystagmus, and the recorded eye movements appeared to be very irregular However the ratio of the slow phase eye speed to the head speed (Gain) proved to change regularly in proportion to the increase of head speed (Fig 7) This result strongly suggests that when the subject try to see a target while they rotate their heads there is a fixed relationship between the slow phase eye speeds of vestibularly and visually-induced eye movements

The ability to pursue a target in the mirror as well as that for visual fixation on a target moving with the head were not correlated with the VOR Gain with mental arithmetic in the dark. Thus the vestibulo-ocular reflex which interacts with the retino-oculomotor reflex should have a Gain that differs from the Gain with mental arithmetic in the dark

Although it has been proved that the vestibulo-ocular reflex is plastic and dependent on the surrounding visual environment (Gonshor & Melvill Jones 1973 Miles & Fuller 1974 Melvill Jones & Davies 1976) the time required for adaptation of the VOR Gain to a new condition depends on the magnitude of the deviance of the physiological relationships of the vestibular and visual stimulations Accordingly antagonism between visual and vestibular stimuli usually results in manifest deterioration of the visual function except for experimental conditions involving extremely long exposure to artificial stimuli (Igarashi et al 1977 Takahashi et al 1978)

The function of the vestibulo-ocular reflex is to keep the gaze spatially fixed during head rotations even without visual information as

found in 'imaginary target in space' in this study. Thus it was suspected that even if the subjects are given contradictory visual and vestibular stimulations, the VOR Gain should always be maintained at unity by the vestibulo-ocular reflex during active head rotations when the modulation of the Gain responsible for the pursuit eye movements is discounted.

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Masahiro Takahashi M.D
Dept of Otolaryngology
Tokyo Women Medical College
10 Kawadacho Shinjuku ku
Tokyo Japan

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INDUCED VESTIBULAR DYSFUNCTION IN SQUIRREL MONKEYS DURING RAPID DECOMPRESSION

Jack P. Landolt, Kenneth E. Money, E. D. L. Topliff,
K. N. Ackles[1] and W. H. Johnson

From the Defence and Civil Institute of Environmental Medicine, Downsview, Ontario and [1]Department of Otolaryngology, University of Toronto, Toronto, Ontario, Canada

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Abstract. The symptoms of postural instability and dizziness associated with decompression sickness could be ascribed to either damage of the vestibular apparatus or to central nervous system damage. However, a histological study of monkeys exposed to decompression reveals that these symptoms primarily result from damage to the vestibular apparatus (unless there are accompanying central deficits). Furthermore, the damage is of a type that causes new bone growth to occlude the otic fluid spaces of the semicircular canals. In some instances, there is sufficient bone growth to render the cristae ampullares as non-functional end organs. Such diminished vestibular function could present a serious threat to the diver.

Normally, man relies on visual, proprioceptive, vestibular and auditory cues to maintain spatial orientation. Underwater, vestibular cues are of primary importance and any dysfunction of the vestibular system, with subsequent unsteadiness ("staggers") and vertigo accompanied by nausea, vomiting and nystagmus not only poses a serious threat to the survival of the diver, but the sequelae may incapacitate him for future employment, both non-diving as well as diving, even if he does survive. The recent increase in diving activities at greater depths, and with different breathing gas mixtures has increased significantly the incidence and awareness of vestibular problems (Caruso et al., 1977; Edmonds et al., 1973; Farmer, 1977; Kennedy, 1973). Kennedy & Diachenko (1975) cite evidence that otologic problems are second in frequency only to joint pain in decompression sickness.

We have investigated vestibular dysfunction, both before and after conditions of rapid decompression, using the squirrel monkey (*Saimiri sciureus*) in simulated diving experiments in a 300 m hyperbaric chamber (Bethlehem Corp., 0.173 m³ capacity). To avoid otic barotrauma, a bilateral myringotomy was performed under light Nembutal® anesthesia the day before the dive. Vestibular function was assessed before the dive and again afterwards, by observation of spontaneous and induced nystagmuses, judgement of the degree of difficulty in walking and standing and the occurrence of any signs of vomiting. Pre-dive and post-dive positional nystagmus in darkness (nose up, nose down, right side down (RSD), left side down (LSD)) and post-rotatory nystagmus in darkness (head erect, RSD, LSD; clockwise and counterclockwise rotations) were recorded by electronystagmography (Barber, 1973). Any pre-dive spontaneous or positional nystagmus contraindicated an animal's participation. Histological study of the brain and temporal bone tissue utilized 30 µm hematoxylin-eosin (H&E) stained celloidin sections (Igarashi, 1966), fixed by intra-arterially perfused 10% formalin under deep Nembutal® anesthesia (≈1 ml/kg body mass).

To date, over 100 monkeys have been decompressed on a diving profile which produced discrete vestibular hits in 35% of attempts (Landolt et al., 1977). Hits were identified by the sudden onset of a spontaneous nystagmus during ascent between 61 meters of sea water (msw) and the surface. When de-

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Masahiro Takahashi M.D
Dept of Otolaryngology
Tokyo Women's Medical College
10 Kawadacho Shinjuku-ku
Tokyo Japan

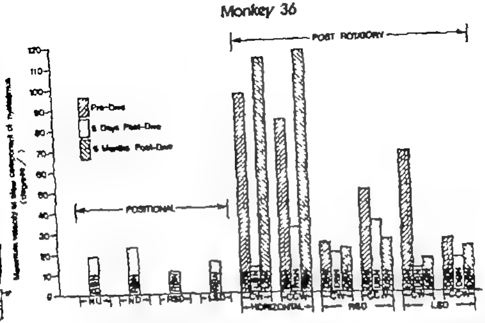


Fig. Bar graph displaying composite electronystagmographic records in Monkeys 36 to time of sacrifice 184 days following successful vestibular hit. Note that positional (or more appropriately spontaneous) nystagmuses are present only at 5 days post-dive: they are absent prior to the dive and 6 months post-dive. Note also the complete recovery of post-rotatory nystagmus at time of sacrifice to horizontal clockwise (*CW*) and counterclockwise (*CCW*) rotations. Caption to Fig. 1D should be referred to for appropriate interpretation of bar graph. *Abbreviations*: *NU ND* = nose up, nose down; *RBN LBN DBN UBN* = right-, left-, down-, up-beating nystagmus.

though reduced throughout, was seldom found in the vestibular perilymph and only occasionally (in traces) on the cupula. Hemorrhage was also commonly found in the vestibular perilymph, particularly along the semicircular ducts in monkeys sacrificed 1 month or less following the dive. A tearing of the endosteum that lines the bony walls (semicircular canals) often accompanied the hemorrhage. Only occasional red blood cells (*rbc*) were found in the vestibular endolymph. (Cochlear damage, though less severe than vestibular damage, takes place with hemorrhage and *pm* occurring together or alone in any scala along any turn.) *These results indicate that the pm can appear without overt hemorrhage.* They also suggest that the *pm* in the vestibular perilymph and cochlear ducts is likely comprised of hemolytic *rbc* and other blood protein exudates (since it appears most often when there are accompanying hemorrhagic incidents). The origin of the *pm* in the endolymph is less clear although its staining properties suggest that it is of proteinaceous origin, e.g. from blood serum or bacteria resulting from an inflammatory reaction.

The most surprising result was the presence of fibrous tissue and new bone growth in the semicircular canals of monkeys that were sacrificed from 1 month to 1 year after the dive. In the 11 monkeys sacrificed 1 month or longer, 9 showed this new bone growth. It appeared either unilaterally or bilaterally, and sometimes several canals were involved (e.g. Fig. 1B). In most cases, only the perilymphatic spaces of the semicircular canals were filled with bone, though *in two monkeys* (e.g. Fig. 1D) the endolymphatic spaces were also totally occluded *so that the semicircular canals were completely functionless*. In two other instances, the new bone growth had progressed to such an extent that ampullae were also invaded by bone and fibrous tissue (e.g. Fig. 1C). When this occurred, the sensory epithe

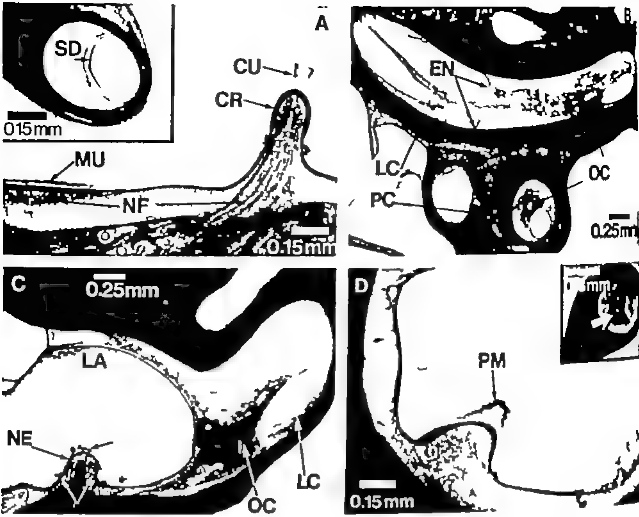
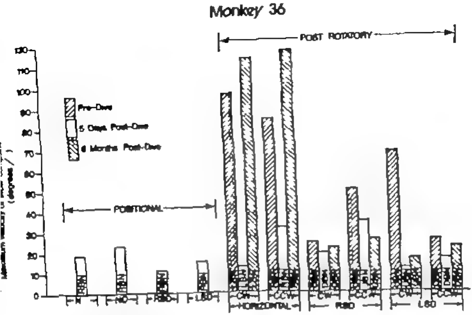


Fig 1 (A) Histology of part of vestibular apparatus in a control monkey illustrating macula utriculi *MU* the crista ampullaris *CR* of the lateral semicircular canal with its cupula *CU* and their innervation *NF* *Inset* Associated semicircular duct *SD* (B) Illustrating new bone growth and fibrous tissue in the perilymphatic spaces of the right posterior *PC* and lateral *LC* semicircular canals in Monkey 24 (379 days post-dive survival) The perilymphatic spaces in the right anterior canal were also invaded by new bone growth the canals on the left side were free of bone The endosteum, *EN* appears to hold the new bone growth and fibrous tissue in place Also shown in both canals are centres of ossification *OC* (C) Illustrating new bone growth in perilymphatic space of left lateral semicircular canal *LC* and its ampulla, *LA* in Monkey 68 (290 days post-dive survival) There is a similar pattern of bone growth in the left anterior semicircular canal and ampul The left posterior canal and those on the right side free of new bone Note the absence of cupula (*dark ar row*) the thin neuroepithelium, *NE* and the areas re gions in the crista which designate missing nerve fibe (*ring-tailed kite arrow*) (cf Fig. 1 A) Centres of ossifi cation *OC* are also shown (D) Darkly-stained precip tated material *PM* in cupula of ampulla in left anterio semicircular canal in Monkey 36 (184 days post-dive su vival) *Inset* New bone growth (*kite arrow*) which h filled the lumen of the semicircular canal and has result in a complete collapse of the S D This monkey had th otic spaces in both left vertical canals and the perilympha tic space in the left lateral canal occluded with new bo growth the canals on the right side were free of bone

compression sickness occurred the monkey was recompressed immediately to twice its depth and then surfaced at the rate of 0.3 msw per minute

Our previous work (Landolt et al 1977) indicated that a granular precipitated material (*pm*) (that stains a deep purple with H&E) was found in all otic fluid spaces in the inner ea (and in particular as an adherent to th gelatinous cupula of the crista ampullaris) I non-dived control animals (3 without myrin gotomies (2 formalin fixed 1 Heidenhain Sus fixed) 2 sacrificed at 3 days 2 at 8–9 days and 2 at 60 days following myringotomies) the *pm*



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lial layer on the crista appeared thin and condensed with no apparent evidence of hair cells (cf Figs 1 A C) (Bone growth did not occur in the otic spaces in any of the control monkeys nor in the cochleae of the dived animals)

Twenty two brains were studied for CNS pathology Of these 8 were from monkeys having behavioral symptoms indicative of both peripheral vestibular and central (mainly spinal) lesions Since a 24 hour survival time beyond the initial insult is essential for morphological changes to become manifest sufficient time did not elapse following the insult to allow for positive identification of CNS pathology in most of these monkeys (6 were sacrificed shortly after the dive) Surprisingly Monkey 40 though surviving only for 150 minutes did show what was interpreted as zones of perivascular edema probably indicative of impending widespread damage that could have involved auditory and vestibular connections in the upper brain stem and hemispheres Monkey 39 which also demonstrated similar behavioral patterns and was sacrificed 1 day post-dive showed small vague focal ischemic lesions some of which involved the cortex of the superior temporal gyrus and underlying white matter and could have interfered with auditory connections The remaining brains were from animals judged to have received discrete peripheral vestibular hits without concomitant CNS vestibular insults Small focal ischemic lesions were identified in 3 of these brains (animals sacrificed 2 days or more following the dive) but none were considered to involve auditory or vestibular connections Thus unless there is widespread accompanying decompression illness vestibular dysfunction appears to be mainly an end organ phenomenon

The results above suggest that during decompression bubble formations occur in the labyrinthine blood vessels which supply the semicircular ducts or more likely in the otic fluid spaces in the inner ear It appears that there are gross disruptions of labyrinthine membranous structures causing both hemorrhage and fluid shifts The sequelae to hemorrhage results in new bone growth which is likely produced by osteoblasts that had differentiated in response to vascular disruptions causing a tearing or irritation of the endosteum that lines the bony wall of the semicircular canal (These canals have an appearance similar to the fibro-osseous labyrinthitis that Schuknecht (1974) refers to as labyrinthine sclerosis) The consequences of fluid shifts are 1) the tearing loose of the semicircular ducts from their bony counterparts (canals) with subsequent blood vessel rupturing and 2) abnormal shifts in endolymph causing in part the agglutination of sediments of *pm* onto the cupula and therefore biasing the neural message that the brain receives

Appropriate test procedures immediately following a dive show that the monkeys possess a vigorous nystagmus In most cases, regardless of position the nystagmus beats in the same direction ('spontaneous nystagmus (Fig 2)) In some instances it is direction changing as if the cupulae have become sensitive to gravity possibly as a result of the adherent *pm* In this regard the clinical features are similar to those resulting from the ingestion of heavy water (Money & Myles 1974) Tests on animals which have been allowed to survive for long periods of time after decompression show that these positional or spontaneous nystagmuses usually disappear (even though there may still be adherent *pm* on the cupula—see Fig 1 D) This is consistent with the view that central compensatory mechanisms regulate and restore over a period of time any such imbalances (McCabe & Ryu 1969)

Although there is often reduced or even complete loss of sensitivity to post rotatory stimuli in one or more canals shortly after decompression there is partial or complete recovery in some monkeys allowed to survive for 2 months or longer (e g see response to horizontal post rotatory stimuli in Fig 2) However if the vertical canals on the same side of the head are completely blocked as a

result of fibro-osseous labyrinthitis (e g. the left anterior (Fig 1 D) and posterior canals in Monkey 36) there is scant likelihood of determining this from clinical diagnostic procedures that rely solely on post-rotatory stimuli (e g. note the similarities in RSD and LSD (vertical canal-stimulating) post-rotatory profiles for pre-dive and 8 months post-dive conditions in Fig. 2) (Technical difficulties prevented use of the more discriminatory caloric vestibular technique for assessing canal function)

This study shows that the damage to monkeys suffering otologic decompression sickness (if not cleared by immediate recompression) is likely to be permanent This means that, weeks or months after suffering inner ear decompression damage if some degree of postural instability develops (or persists) or if visual blurring with head movements develops or if caloric vestibular tests show reduced or no responses these developments can be understood on the basis of semicircular canal paresis caused by new bone growth in the canals and without recourse to central etiologies Such damage to humans is fraught with probable grave consequences should they return to future diving It is evident that the entire diving community should be made aware of the hazards to the inner ear which can result from diving. Furthermore appropriate damage prevention procedures with routine clinical screening and testing should be a requirement for any agency that is regularly involved in diving activities.

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ZUSAMMENFASSUNG

Instabile Körperstellung und Schwindel als Symptome der Dekompressionskrankheit konnten entweder durch Schädigung des Vestibularisapparates oder durch Läsionen im Zentralnervensystem begründet werden Jedoch in einer Untersuchung an Affen die einer Dekompression ausgesetzt worden waren, fanden sich im wesentlichen Schädigungen am Vestibularisapparat. Diese Schädigung hat den Charakter eines fibrösen Verschlusses der Bogengänge mit neuem Knochenwachstum an Affen die nach Zeiten von einem Monat bis zu mehr als einem Jahr nach dem Tauchen geopfert wurden. Solch eine Schädigung des Vestibularisapparates konnte eine ernsthafte Bedrohung des Tauchers darstellen

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Dr J P Landolt
DCIEM
P.O Box 2000
Downsview
Ontario
M3M 3B9 Canada

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ovocation tests The results of McLean et . (1976) did not convincingly show such an fect of isoprenaline Placebo was not used in e two trials and Jorde et al (1975) did not escribe the technique for allergen provocation Moreover the results of Jorde et al 975) contrasted with an unpublished placebo-controlled double-blind crossover study erformed in our laboratory on 20 patients ith perennial allergic rhinitis due to house ust, who were treated *orally* with terbutaline or ? weeks (Svensson 1974) In this study placebo was as good as terbutaline (5 mg×3) n diminishing the patients nasal troubles However in another unpublished pilot study of the prophylactic effect of *nasal* application of terbutaline (0.5 mg to each nasal cavity) to 9 asymptomatic patients with hay fever rhinomanometric registrations of nasal patency before and after nasal provocations showed terbutaline to be superior to placebo in diminishing nasal obstruction due to challenges (Hegardt & Svensson 1977)

One purpose of the present study was to gain further experience of the antianaphylactic effect of terbutaline A second purpose was to evaluate the effect of a new β-adrenoceptor agonist KWD 2131 which in animal experiments had shown the same antianaphylactic action but lower cardiovascular and bronchodilating effects than terbutaline (Sorenby 1977) Based on these data, KWD 2131 seemed to offer some advantages as a drug in nasal allergy and a comparison between the two drugs in human experiments should be of interest In such a study repeated nasal allergen provocations in the same patients had to be done with hyposensitization or priming effect i e decreased or increased reactions as possible consequences Thus a third purpose of the study was to reveal a changed reactivity of the nasal mucosa due to the experiments

MATERIAL AND METHODS

The trial was conducted over a pollen-free period of 3 months, January–March 1978

A total of 13 patients (7 males and 6 females aged 17–29 years average 25 years) with seasonal allergic rhinitis were included in the trial. All of them were asymptomatic *(out of season)* but sensitive to pollen as shown by positive skin test and positive provocation test and they had suffered from hay fever during the last two seasons at least. Any patient exhibiting nasal polyps or asthmatic symptoms was excluded

The preparations under investigation were two β-adrenoceptor stimulating drugs terbutaline sulphate (Bricanyl® provided by AB Draco subsidiary to AB Astra, Sweden) dihydroxyphenyl ethanol sulphate (KWD 2131 AB Draco) and placebo (isotonic saline solution) The dosage of terbutaline was 0.5 and 5 mg and for KWD 2131 1.5 and 5 mg to each nasal cavity The drugs were applied locally as nose drops from a pipette in two equal portions (2×0 08 ml) to each nasal cavity with a time interval of 2 minutes between the portions. During the drug application the subject was sitting with his/her head tilted backwards and laterally for administration against the lower turbinate Placebo was delivered in the same volume and manner as terbutaline and KWD 2131 After each application the subject was asked for any taste of drug for evaluation of nasal and/or pharyngeal deposition. Experiments with suspected pharyngeal deposition of the drugs were put off to another day When used the drugs had the same temperature as the testing room i e 22–23°C In the meantime they were stored in a refrigerator (4–8°C)

The nasal provocations were performed with the disc method (Okuda, 1977) which involves small paper discs (squares with sides of 8 mm) soaked with 0 05 ml allergen solution being attached to the *anterior part of one lower* turbinate At each visit, the less swollen turbinate was selected for provocation. First two things were determined the suitable concentration of the allergen solution and the duration of provocation for each volunteer to induce a positive provocation reaction in the

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EFFECTS OF TOPICAL USE OF β-ADRENOCEPTOR STIMULANTS ON NASAL MUCOSA RHINOMANOMETRIC EVALUATIONS IN EXPERIMENTS WITH TERBUTALINE AND KWD 2131

II *Nasal Provocation Tests with Hay Fever Patients in Asymptomatic Period*

Gunnar Svensson

From the Department of Otorhinolaryngology University Hospital Lund Sweden

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Abstract The effects of the β-adrenoceptor stimulating drugs terbutaline (Bricanyl®) and KWD 131 on the increase in nasal airway resistance induced by pollen extracts have been studied in a placebo-controlled double blind crossover trial of 13 asymptomatic (out of season) patients with hay fever For objective evaluation of the nasal airway resistance posterior rhinomanometry has been used The rhinomanometric measurements indicated an antianaphylactic effect of terbutaline and KWD 131 but rhinoscopy and the patients own opinion could not establish such an effect The design of the trial made it possible to establish whether or not an increased or a decreased nasal obstruction due to repeated challenges occurred Despite 6 preceding nasal provocations in each participant neither increased ("priming effect") nor decreased (hyposensibilization) reaction was detected in the following challenge

Extrinsic asthma hay fever and the wheal and flare reaction in skin may all be considered as local manifestations of the immediate (Type I) anaphylactic reaction As early as over four decades ago Tuft & Brodsky (1936) showed that adrenalin suppressed the reagin-dependent wheal and flare reaction in human skin and Schild (1936) demonstrated that adrenalin reduced the immunologic release of histamine from guinea pig lung tissue Subsequently a variety of sympathomimetic amines were shown to be capable of inhibiting the immunologic release of mediators from rat mast cells guinea pig lung as well as human lung leukocytes and nasal polyps (Assem & Schild 1969 Orange et al 1971 Kaliner et al 1973 Kaliner & Austen 1974 Sörenby 1974 *a b* Kaliner & Austen 1975 Lichtenstein 1975 Sörenby 1975) Experimental data have demonstrated that β-adrenoceptor stimulating agents increase the tissue level of cyclic AMP Moreover an inverse dose–response relationship is stated between the tissue concentration of cyclic AMP and the capacity for immunologic induction of the secretion of chemical mediators However the role of cyclic AMP in histamine release has been questioned recently (Diamant et al 1978)

Clinical studies also support the usefulness of β-adrenoceptor stimulation for blocking the immediate hypersensitivity reactions Thus, Shereff et al (1973) and Lamkin et al (1976) could diminish skin test reaction in atopic subjects after iontophoretic application of isoprenaline The same effect was also documented for terbutaline alone (Grönneberg et al 1978) and for both terbutaline and KWD 2131 (Hegardt & Arner 1978) Systematically administered adrenalin and isoprenaline to allergic patients reduced the wheal and flare reaction (Kram et al 1975) This was also shown in rhesus monkeys after administration of salbutamol (Perper et al 1972) Furthermore chronic urticaria has been successfully treated with terbutaline (Kennes et al 1977)

Two published studies concerning the effect of β-adrenoceptor stimulants on nasal allergic symptoms have reported contradictory results In a study by Jorde et al (1975) nasal application of fenoterol and salbutamol had a prophylactic as well as curative effect in nasal

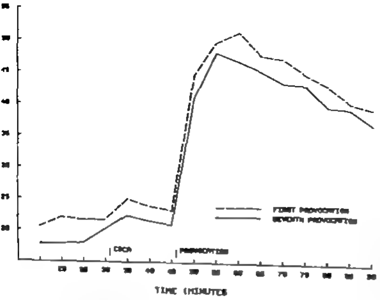

Fig. 2. Nasal airway resistance versus time. Mean values of 13 patients with allergic rhinitis.

hallenges. This examination was performed after the rhinomanometric evaluation. Also he patients' subjective opinion of nasal symptoms was recorded on a 0–3 scale. The pulse requency was regularly determined before rug application and before rhinomanometry. land tremor before and after drug administra on was also recorded on a 0–3 scale.

The test procedure is given schematically s follows:

nasal challenge
drug or Coca drug application
subjective evaluation
pulse frequency determination
posterior rhinomanometry
anterior rhinoscopy
hand tremor estimation

Rhinomanometry

The nasal airway resistance for both nasal avities together (total nose) was determined uring spontaneous breathing as posterior hinomanometry in a sitting position, using he technique of Broms et al. (1979) for evaluation of the results. Thus an angle v as a measure of nasal airway resistance was calculated. Details have been given in a previous study (Svensson et al. 1979). Increasing values of v denote increasing nasal airway resistance.

The differences in angle v_2 were analysed statistically with Student's paired t test.

Because of acclimatization of the subject to the experimental situation no statistical analyses were done until after 30 minutes stay in the testing room.

The study was approved by the Ethical Committee of the University of Lund and informed consent was obtained from all patients.

RESULTS

Fig. 1 shows the changes in nasal airway resistance expressed as the angle v under different conditions (adaptation, after treatment with terbutaline and KWD 2131, placebo, and after nasal provocation). It is obvious that pretreatment with the actual drugs caused only minor changes in nasal resistance. This was also in agreement with the patients' subjective opinion and the rhinoscopic findings.

The changes in nasal airway resistance re-

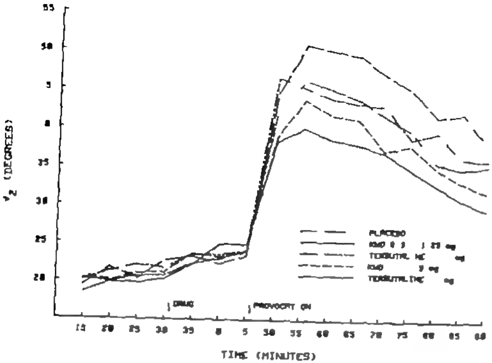

Fig 1 Nasal airway resistance versus time Mean values of 13 patients with allergic rhinitis

nasal mucosa For each person the same allergen concentration (1 100 in Coca s solution (Philips 1967) for all subjects and 1 10 for 2 subjects not reacting on 1 100) and duration of provocation (3 or 5 min) were used in all experiments

The study included 7 visits with nasal challenges—5 preceded by treatment with terbutaline KWD 2131 and placebo in a double blind crossover manner for evaluation of antianaphylactic effects of the substances and 2 (the first and last) provocations without any pretreatment with drugs for determinations of nasal hyposensibilization or priming effect On these two occasions instead of treatment with drugs a disc soaked in Coca s solution was applied to the anterior part of the lower turbinate for 4 min This was done 15 min before the succeeding allergen provocation in order to evaluate the mechanical effects of the disc

The interval between each provocation was 1 or 2 weeks The drugs were tested in a randomized order Each subject was interviewed and medically examined (rhinoscopy for evaluation of nasal obstruction and secretion pulse frequency determination and hand tremor examination for evaluation of side effects) before entry into the trial as well as before during and after each testing All subjects were asymptomatic as to nasal symptoms before each provocation and free from drugs

The stock solution of allergen (1 10) was stored in a refrigerator throughout the experiment and new dilutions were prepared each week These were also stored in a refrigerator until 45 min before using The effects of the provocations were observed for 45 min Eleven patients were challenged with timothy (*Phleum pratense*) solution one with broadleaf tree (*Alnus glutinosa Betula verrucosa* and *Corylus avellana*) solution and one with mugwort (*Artemisia vulgaris*) solution

The nasal patency was evaluated with posterior rhinomanometry The first rhinomanometric determination was done after 15 minutes adaptation to the testing room and repeated every 5 min The preparations were administered after 30 min and the nasal challenges carried out after 45 minutes stay in the testing room The condition of the nose was also recorded rhinoscopically as to the presence of oedema and secretion on a 0–3 scale (0=no symptoms 1=mild 2=moderate 3=severe) before and every 5 min after application of the preparations and the nasal

Table I *Comparison of the increase of nasal airway resistance measured as v_3, relative to the 5 min value*

Time / Drug compared	50	55	60	65	70	75
	Increase in					
Placebo Terb 5 mg	7.7*	12.3**	13.0**	13.0**	11.6**	11.8**
Placebo Terb 0.5 mg	−1.4	6.3	6.9**	6.8*	4.7	7.0 s.
Placebo KWD 2131 5 mg	6.3	7.9*	9.1**	9.0**	10.5	7.9*
Placebo KWD 2131 1.25 mg	2.8	5.3 s.	5.6 n.s.	6.2 s.	5.9*	5.9 n.s.
KWD 2131 5 mg Terb 5 mg	1.4	4.4 s.	3.9	4.0 n.s.	1.0 n.s.	3.9 s.
Terb 0.5 mg KWD 2131 1.25 mg	4 n.s.	−1.1 s.	−1.3 n.s.	−0.6 n.s.	1.2 s.	−1.1 s.

not significant $p<0.05$ $**=p<0.01$

In therapeutic oral doses the conventional β-adrenoceptor stimulants often cause side effects such as tachycardia and tremor. In animals (Sorenby 1977) KWD 2131 exhibited an antiallergic effect similar to that of terbutaline but less pronounced cardiovascular and bronchodilating effects. A clinical study of healthy volunteers (Pegelow & Strandberg, 1978) including measurements of heart rate, blood pressure and tremor showed terbutaline to be about 10 times as potent as KWD 2131 with respect to cardiovascular effects. The profile of action for KWD 2131, i.e. an appreciable antianaphylactic effect in concentrations causing negligible bronchodilating and cardiovascular effects, makes the substance an interesting tool for clinical evaluation of antianaphylactic effects.

As no previous study had been performed with terbutaline and KWD 2131 applied to the nose, various doses had to be evaluated with respect to antianaphylactic effect. The low dose of terbutaline (0.5 mg to each nasal cavity) was based on experiences from ordinary treatment of asthmatic patients (McPhillips 1977) and from a pilot study on patients with allergic rhinitis (Hegardt & Svensson, 1977). The high dose (5 mg) was chosen considering the fact that no obvious side effects were observed after inhalation of 5 mg terbutaline (Bennis & Svedmyr 1977). The low dose of KWD 2131 (1.25 mg to each nasal half) was previously shown to have doubtful or weak antiallergic properties (Hegardt et al., 1978, Pegelow & Strandberg 1978).

Application of discs is one of the methods for nasal provocation tests. According to Okuda (1977) the disc method is suitable without disturbing nasal reactions. However, he based his opinion on rhinoscopy only. In the present study, using rhinomanometry, it was found that the nasal airway resistance increased but returned to about the same value as before the application of the disc within 10 min after the extraction of the disc. It did not differ from administration of placebo in changing the nasal resistance. Thus, our method for application of disc seems to be suitable for nasal provocation.

The nasal airway resistance after allergen challenges, evaluated with posterior rhinomanometry, decreased after pretreatment with terbutaline and KWD 2131 compared with placebo (Fig. 1). The differences between terbutaline 5 mg and placebo as well as between KWD 2131 5 mg and placebo were statistically significant (Table I), suggesting an antianaphylactic effect of the substances at these dosages. Such an effect, but less distinct, was also observed after terbutaline 0.5 mg (Table I). Comparisons between the rhinomanometric results obtained after terbutaline 5 mg and KWD 2131 5 mg showed no significant differences (Table I). This ought to signify a comparable antianaphylactic effect of the two substances at the given dosage. These results are in agreement with those obtained in experiments on animals (Sörenby 1977) and also with the results on skin reactions (Hegardt & Arner 1978).

fore and after application of a paper disc with Coca s solution to the lower turbinate are seen in Fig 2 This treatment of the nose resulted immediately in an increased nasal airway resistance These increases are small but statistically significant However the mean value was gradually reduced to almost the preapplication value 10 min after extraction of the disc The rises in nasal airway resistance after application of the disc were also compared with those induced by administration of placebo into the nose No statistically significant differences in the capacity of changing nasal airway resistance were revealed between the two methods The participants experienced no nasal changes during the experiments and the rhinoscopic examination revealed no changes

The rhinomanometric determinations of nasal patency just before provocation minute 45 (Fig 1) did agree fairly well between the treatment groups

The nasal provocations at minute 45 were followed by marked increases in nasal air flow resistance Compared with the values before provocation all these increases in nasal airway resistance were highly statistically significant Also the patients subjective opinion and the rhinoscopic findings supported the rhinomanometric determinations

The nasal provocation without pretreatment (Fig 2) or after pretreatment with placebo (Fig 1) caused the most pronounced increases in nasal airway resistance Pretreatment with KWD 2131 1 25 mg terbutaline 0 5 mg KWD 2131 5 mg and terbutaline 5 mg to each nasal cavity were in the given sequence followed by gradually diminishing increases in nasal resistance Thus the best protection was given by terbutaline 5 mg and KWD 2131 5 mg Compared with placebo pretreatment these doses of the two substances caused diminished increases in nasal airway resistance after nasal provocation (Table I) This effect was statistically significant A reduced increase was also found after terbutaline 0 5 mg but this effect was less conspicuous

The rhinoscopic findings and the patients own opinions about the symptoms after nasal challenges revealed no evident differences between pretreatment with terbutaline, KWD 2131 or placebo

Increased pulse frequency and hand tremor were found in all patients when 5 mg terbutaline was administered to each nasal cavity One patient was also troubled by tremor of the hands after administration of the low dose (0 5 mg) of terbutaline and the high dose (5 mg) of KWD 2131 In another 2 patients tremor was suspected after application of the low dose of terbutaline

A comparison of the results obtained at the first and last nasal provocation—both without pretreatment with any drug—gave an evaluation of the effect of repeated nasal challenges It was obvious that six earlier provocations neither diminished nor increased the airway resistance in the following (the seventh) nasal provocation (Fig 2) Moreover six challenges did not change the nasal reactions after application of a disc without allergen i.e. non-allergic stimulus (Fig. 2)

DISCUSSION

Sympathomimetic amines inhibit antigen-induced release of histamine in many tissues (Assem & Schild 1969 Kaliner et al 19[illegible] Sörenby 1974*a* Kaliner & Austen 197[illegible] The inhibiting effect of histamine release is blocked by propranolol indicating involvement of β adrenoceptors The β-adrenoceptor stimulants isoprenaline orciprenaline and terbutaline sulphate show inhibition of anaphylactic histamine release in the guinea pig lung in the same concentrations as they evoke tracheorelaxation (Sörenby 1975) Therefore allergen provoked bronchoconstriction does not seem to be a suitable model for the examination of the antiallergic effect of these drugs The nasal mucosa, however with no or only a few β adrenoceptors in the vessels (Svensson et al 1979) should be a better organ for an evaluation of this effect

treatment with drugs the application of a disc soaked with Coca's solution and thereafter provocation with allergen. Comparing the reactions after the application of a disc with Coca's solution no differences between the two visits were found indicating an unchanged reactivity to environmental factors. Nor were there any differences in response to allergen. Thus in the present trial neither priming effect nor nasal hyposensibilization could be demonstrated.

ZUSAMMENFASSUNG

Die Wirkung der β-adrenoceptorstimulierenden Substanzen, Terbutaline (Bricanyl®) und KWD 2131 in bezug auf die Erhöhung des Atemwiderstandes der Nase durch Pollenextrakte war Gegenstand eines placebokontrollierten doppelten Blindversuches an 13 asymptomatischen Patienten mit Heufieber. Zur objektiven Bestimmung des Atemwiderstandes der Nase wurde die posteriore Rhinomanometrie angewandt. Die rhinomanometrischen Messungen zeigten einen antianaphylaktischen Effekt von Terbutaline und KWD 2131 an, aber sowohl Rhinoskopie als subjektive Empfindung der Patienten konnten einen solchen Effekt nicht bestätigen.

Die Anordnung der Untersuchung machte es möglich festzustellen, ob eine verstärkte oder geschwächte Schwellung der nasalen Schleimhaut aufgrund wiederholter Untersuchungen eintrat oder nicht. Trotz 6 vorangegangener Untersuchungen an jeder Versuchsperson wurde in der folgenden Untersuchung weder eine verstärkte („priming-Effekt") noch geschwächte (Desensibilisierungs-)Reaktion festgestellt.

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80	85	90
10 4	12.8	10 1
? 7 n.s	6.5 n.s	? 7 n.s
7 4	9 7	7 0*
6 6 n s	7.8 n.s	?.3 n.s
3 0 n.s	3 1 n.s	3 1 n.s
3.9 n.s	1.3 n s	0.6 n.s

In contrast to the lower dose (0.5 mg) of terbutaline that of KWD 2131 (1 25 mg) did not show any antianaphylactic effect compared with placebo (Table I) This agrees with the results from studies on asthmatic patients (Hegardt et al 1978 Pegelow & Strandberg 1978) but is on the other hand somewhat surprising with respect to the comparable anti anaphylactic effect demonstrated in experiments on animals (Sörenby 1977) and with the higher dose in this study Different slopes of the dose–response curves for terbutaline and KWD 2131 or a displacement to the right for that of KWD 2131 could perhaps offer an explanation for this divergence

The present results are in accordance with those drawn from a pilot study (Hegardt & Svensson 1977) using topical terbutaline in hay fever patients Jorde et al (1975) in their study with fenoterol and salbutamol also found a prophylactic effect of these substances in allergic rhinitis judging from their graphical illustration the effect of fenoterol seemed to be more pronounced than that of terbutaline and of KWD 2131 in the present trial In the study by McLean et al (1976) local application of isoprenaline prior to ragweed challenges produced either a variable inhibition of the increases in nasal airway resistance or no protection at all The disparity of the results could be due to methodological variations Jorde et al (1975) have not given details of the provocation procedure and McLean et al (1976) have used a provocation technique with an expected increase in the nasal airway resistance of about 200% Of course a weak antiallergic effect cannot neutralize a very strong provocation reaction. In the present study with a demonstrable antiallergic effect of the two β_2-adrenoceptor stimulants the increases in angle v_2 after challenge were about 100% corresponding to an increase in nasal airway resistance of 100–150% ($R = 5 \tan v_2$).

The antianaphylactic effects of terbutaline and KWD 2131 in this study are based on rhinomanometry The clinical relevance of these effects is fundamental and the rhinoscopic findings the patients own opinion and any side effects must be evaluated In this study the rhinoscopic findings and the patients own opinions did not reveal any obvious differences between the substances For a better evaluation of the antiallergic effect a clinical trial with registration of the subjects symptoms on diary cards during a pollen season might be of interest However the side effects of administering 5 mg terbutaline to each nasal cavity found in the present study preclude further investigations with terbutaline With respect to this KWD 2131 should be preferred

The design of the present study made it possible to estimate the development of priming effect or nasal hyposensibilization. Priming effect is a phenomenon characterized by an increased reactivity of nasal or bronchial mucosa following repeated exposures to allergen Moreover when the priming effect is established environmental factors which normally do not produce reactions in the shock organ can then induce such reactions (Connell 1969) Hyposensibilization is a phenomenon in the opposite direction i e a decreased reactivity of the challenged mucosa following repeated exposures to allergen. Nasal hyposensibilization due to repeated nasal treatment with different antigenic preparations has been described by several authors (Gleich & Yunginger 1975 Mehta & Morrison Smith 1975 Deuschl & Johansson 1977) In the present study the first and last visit for the volunteers included without pre

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COMPUTER AIDED RHINOMETRY

A Research Rhinometer for Clinical Trial

P Cole O Fastag and V Niinimaa

From the Gage Research Institute University of Toronto Toronto Ontario Canada

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Abstract The effects of several variables on work of nasal breathing and nasal resistance were investigated. Sampling of pressure and flow of nasal respiratory air was performed by posterior rhinometry every 20 msec with microprocessor and standard respiratory laboratory equipment. This technique enables work of nasal breathing and mean nasal resistance to be determined on line. The relationship between work·litre^{-1} and ventilation was always near-linear and its slope is the dimensional equivalent of resistance. Slope and linearity remained constant for several hours at time and were unaffected by moderate hyperventilation, exercise or change in breathing pattern. The slope of the line—but not its linearity—was altered by vasoactive medication, exercise and nasal obstruction. Mean nasal resistance determined by this technique may be employed as a measure of nasal obstruction, its stability and independence from the moderate changes in breathing pattern which occur spontaneously suggest useful clinical application.

More than 20 different methods for measurement of nasal obstruction in terms of resistance (R) are in current use and many others have been described in the literature. For our investigations, which include measurement and comparison of aerodynamics at flow resistive segments of the airways during respiration, we found none of the existing methods of airway resistance measurement to be suitable. The curvilinear relationship between pressure and flow, and the absence of an agreed definition of nasal resistance compounded our difficulties in finding an acceptable resistance method by which to compare the laminar aerodynamic condition of the pulmonary airways with the turbulent ones of the nose and larynx. The combination and comparison of unequal nasal cavities presented further difficulties in the use of currently used resistance methods.

As an alternative we chose work of breathing (Butler 1960) and mean resistance as our pressure–flow relationships, values which we obtained from pressure and flow measurements every 20 milliseconds throughout each respiratory cycle. This departure from established methods was facilitated by the advent of the inexpensive microprocessor which we interfaced with standard respiratory laboratory pressure and flow recording equipment for rapid sampling and calculation.

A series of posterior rhinometric studies was made to determine the feasibility of measuring obstruction to respiratory airflow in terms of work of breathing. Our published results show that in individual normal subjects and nasally obstructed patients during ventilation at rest, work of nasal breathing remains constant for periods of several hours and work of nasal breathing is increased in obstructed patients (Cole et al. 1979).

In the present communication, further experiments are described which demonstrate the effects of altered ventilation and of nasal obstruction on work of nasal breathing.

METHODS

Subjects Normal subjects were selected from laboratory personnel and other volunteers. They had no history of respiratory infection during the preceding 3 weeks and no evidence of cardiac, pulmonary or obstructive nasal disease on clinical examination. The results of their pulmonary function tests were normal

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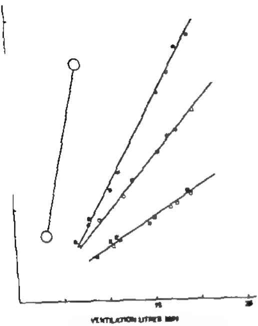
Gunnar Svensson M.D
Dept of Otorhinolaryngology
University Hospital
S-221 85 Lund
Sweden



2 Work litre at different ventilation rates measured in *A* normal subject *B* patient with mild perennial ... *C* patient ... th structural nasal obstruction, *D* ... tient with acute allergic rhinitis.

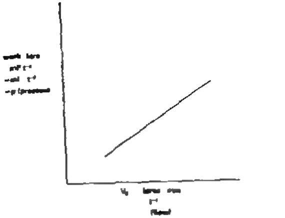


Fig 3 Work litre is dimensional equivalent of mean pressure. Ventilation is mean flow Slope is mean resistance.

$$\frac{P}{\dot{V}} = \frac{\text{ml} \quad l^{-1}}{l \quad l^{-1}}$$

$= \text{ml}^{-1} l^{-1}$

$= R$ (resistance)

...ection indicate a nearly linear relationship ...etween nasal work litre and ventilation ...hich may be extrapolated close to the origin ...f the axes Fig. 2. This impression was con...rmed by statistical analysis of 66 series of ...esults ($\bar{r}$ = 0 98 ± 0 02 S D)

This linearity was found in all normal sub...ects with hyperventilation exercise and ...asoactive nasal medication and in patients with nasal obstruction of a mucovascular or a fixed structural type (Fig 2) Slope and linearity remained constant in an individual subject for as long as 6 hours (Fig. 1) and were independent of moderate changes in breathing pattern when this was altered whether deliberately (Cole et al 1979) or spontaneously Age did not change the degree of linearity

Examination of our units shows that work litre is a dimensional equivalent of pressure and by means of our 20 msec sampling we measure mean transnasal pressure throughout 5 or 10 respiratory cycles Ventilation in litres min is mean flow and from this pressure–flow relationship mean nasal respiratory airflow resistance can be derived (Fig. 3) Measurements of resistance at different ventilation rates remain fairly constant because the relationship between pressure and flow is almost linear over the range of measurement and the line may be extrapolated to near the origin The intercept on the vertical axis is always negative its increase with increasing slope of the line is consistent with projections of the near-linear portions of a series of exponential curves In normal subjects resistance is about 20% higher at 20 l min than at 10 l min ventilation Thus resistance values obtained from an individual subject within the resting range of ventilation show little variation.

We may therefore by means of our computer assisted technique obtain an on-line assessment of nasal obstruction in terms of work of breathing or of resistance and although these experiments were performed for their physiological interest as part of a series on the flow resistive segments of the respiratory airways the results suggest a possible clinical application. The nasal work litre −ventila

Patients Patients were referred by medical practitioners for assessment of obstructive nasal disease

Observations Five sets of observations were made

(1) The relationship between ventilation in litre min^{-1} and work of nasal breathing in Joules $litre^{-1}$ (also Joules $breath^{-1}$ and Joules min^{-1}) was determined in 25 normal subjects by posterior rhinometry at rest and at different levels of moderate exercise on a cycle ergometer Each subject was tested on two occasions and a test consisted of 10 observations at ventilation rates varying between resting values and 40 litres min^{-1} Each observation was obtained from a 5-breath sample A total of 50 tests and 447 usable observations was produced

(2) Eight normal subjects below the age of 25 and 8 above the age of 50 were examined in a similar manner to (1) during voluntary hyperventilation at rest.

(3) One normal subject was examined as in (2) 3–4 observations were made each hour for 6 hours at varied ventilation rates

(4) Two normal subjects were examined as in (2) 5 min after application of a vasoactive nasal spray to each nasal cavity (*a*) 1% histamine acid phosphate (*b*) 0 1% xylometazoline hydrochloride

(5) Several patients who complained of nasal obstruction were examined as in (2)

Experimental technique equipment and data handling have been described in detail previously (Cole et al 1979)

RESULTS

Normal subjects with exercise The linear regressions of work $litre^{-1}$ on ventilation from 50 series of experiments with 25 subjects give a mean correlation coefficient of 0 98± 0 02 S D and an intercept of −0 10 Joules $litre^{-1}$

The relationships between ventilation and work $breath^{-}$ and work min^{-} (power) were markedly exponential

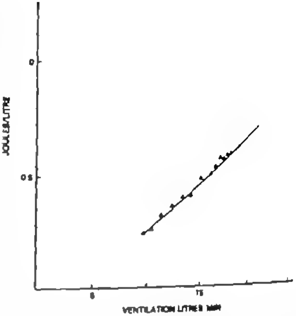


Fig 1 Work litre at different ventilation rates measured at intervals during a 6-hour period in an individual subject Slope and linearity are maintained.

Normal subjects with hyperventilation at rest Analysis of results from the younger and the older groups of 8 normal subjects each gives a mean linear correlation coefficient of 0 98±0 02 S D

Normal subject observed for 6 hours Twenty observations show that both the linear relationship and constant slope are maintained throughout this 6 hour period (Fig 1)

Normal subjects with vasoactive medication The linear relationship is maintained and the slope of the line is made steeper or shallower after application of histamine or xylometazoline respectively

Patients complaining of nasal obstruction Each produced an above normal increase in slope of the regression line though linearity was maintained (Fig 2) (A) Normal (B) Mucovascular disease (perennial rhinitis) (C) Chronic obstruction due to fixed structural abnormality (D) Acute obstruction due to allergic rhinitis

DISCUSSION

In each of more than 200 series of experiments graphical representation and visual in

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GROWTH HORMONE AND PROLACTIN PRODUCTION IN HUMAN PITUITARY TUMOURS IN ORGAN CULTURE

Matti Anniko, Sigbritt Werner[a] and Jan Wersäll

From Department of Otolaryngology, Karolinska Sjukhuset and King Gustav V Research Institute, Stockholm, and [a]Department of Endocrinology, Karolinska Sjukhuset, Stockholm, Sweden

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Abstract. Human pituitary adenomas, when preserved as organ cultures, do not lose their function of synthesizing and releasing hormones. Thus, growth hormone producing adenomas have been cultured for 35 days and prolactinomas for 14 days with maintained hormone hypersecretion, >30 000 pmol/l/48 h of growth hormone and 15 000 µg/l/48 h of prolactin. Preserved morphology/ultrastructure was documented in these specimens. Following repeated renewal of the entire nutrient solution, less than 1/10 of the initial hormone content remained after the first change, indicating an active hormone production/release into the culture medium as hormone concentration rose to previous levels. The growth hormone producing cells could be stimulated by TRH and LH-RH in the concentrations [illegible] and 10 g/l respectively, indicating the same pathological reactivity in vitro as in vivo. Prolactin synthesis in vitro paralleled that in vivo as reflected by serum prolactin levels. Growth hormone synthesis in vitro paralleled the clinical activity of the disease, but the plasma hormone levels in patients with acromegaly did not do so.

We have recently analysed cell characteristics/cell survival after explanting human pituitary adenomas to in vitro conditions, and concluded that a high degree of stability was obtained during the first weeks in culture (Anniko et al., 1979). Recently this has also been reported by Lipson et al. (1978) who used radioimmunoassay methods to measure multiple hormones in the culture medium.

Organ culture offers a simplified system for the study of normal or abnormal pituitaries in the absence of external hormonal or neurogenic control factors. However, there are few such published studies (Skyler et al., 1977; Goodyer et al., 1977). Peillon et al. (1972, 1975) described how somatotrophic pituitary adenomas maintained cytologic differentiation in vitro. These authors were able to correlate morphology to hormonal secretion.

In the present study, the release of anterior pituitary hormones—growth hormone (GH) and prolactin (PRL)—into the culture medium was analysed following *en bloc* (organ) culture of both endocrinologically functioning (GH and PRL producing adenomas) and non-functioning ("chromophobe") pituitary neoplasms. A combination of morphological and biochemical studies of pituitary secretion was undertaken. The tissue was incubated with substances which previously in vivo had caused stimulation of GH and PRL secretion.

Hormone secretion rates in vitro were compared with clinical activity and GH and PRL levels in vivo.

PATIENT MATERIAL AND METHODS

Patients

The investigations of the patients prior to surgery are summarized in Table I. Nine patients were operated upon by the trans-anthrosphenoidal approach and one (no. 6) by the transfrontal route.

Methods

I. *Incubation procedure*

The technique for organ culture of pituitary adenomas has been described in detail by Anniko et al. (1979, in press). After removal of

tion ratio (resistance) in an individual resting subject or a nasally obstructed patient in comfortable and stable ambient conditions remains constant for several hours (Fig 1) (Cole et al 1979) and this resistance is scarcely affected by *the spontaneous changes which* occur in breathing pattern or ventilation Thus the computer assisted posterior rhinometric technique we have described may be readily employed for clinical assessment of nasal obstruction without restriction of a resting subject s spontaneous breathing pattern Furthermore reduction of resistance by vasoconstrictor medication demonstrates the magnitudes of the reversible mucovascular component and the residual fixed component

Our research equipment has been duplicated by interfacing a AIM no 65 microprocessor (dedicated by Rockwell International Microelectronic Devices Anaheim California USA) with standard respiratory laboratory pressure and flow measuring equipment to produce a 20 *character alphanumeric printout* and readout This equipment is now being evaluated in a clinical setting and the additional experience may enable us to establish useful baseline values

ZUSAMMENFASSUNG

Die Einwirkungen verschiedener Varianten auf die Atmung durch die Nase und auf den Widerstand in der Nase werden untersucht Die Untersuchung von Druck und Strom der Atmungsluft in der Nase erfolgt mit 20 md. *Messung in der hinteren Nase mit einem Mikroprozessor* und Standard-Atmungs-Labor Apparatur Diese Technik erlaubt eine graphische Darstellung der Atmungsenergie durch die Nase und des mittleren Widerstands in der Nase Es zeigte sich, daß das Verhältnis zwischen Energie/Liter und Ventilation fast immer geradlinig war und die Kurve das dimensionale Äquivalent des Widerstands angibt. Diese Geradlinigkeit blieb während einer bestimmten Zeitdauer mehrere Stunden lang konstant und von *mäßiger Schnellatmung, körperlicher Bewegung* oder wechselndem Atemrhythmus unbeeinflußt. Die Kurve, aber nicht die Geradlinigkeit wurde durch vasoaktive (die Blutgefäße aktivierende) Medikamente und Obstruktion in der Nase verändert Die Tatsache daß der mittlere Widerstand in der Nase der mit Hilfe dieser Technik ermittelt wird zur Feststellung und Messung von Obstruktion in der Nase verwandt werden kann deren Stabilität und Unabhängigkeit von mäßigen und unwillkürlich vorkommenden Veränderungen des Atemrhythmus, legt ihren Wert für eine gute klinische Verwendung nahe

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P Cole M.D
The Gage Research Inst
Toronto
Ontario
Canada

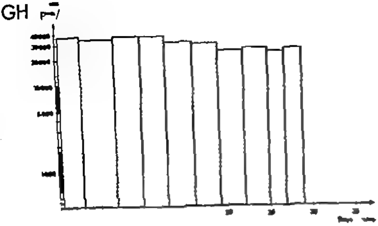

Fig. 1. Continuously high GH production from adenoma tissue (case no. 3) during 29 days in vitro.

renewed every 2–4 days (culture dishes) or after 7 days (culture flasks).

II. *Hormone assays*

GH was measured by a double antibody radioimmunoassay technique according to Cerasi et al. (1966) and PRL using a radioimmunoassay kit (CEA IRE SORIN). The various components as such and the culture media as a whole were screened for GH and PRL prior to use with negative results. In organ cultures preserved in culture dishes the entire culture medium was changed and analysed for hormone content, whereas probes of 0.3–0.5 ml were regularly drawn from culture flasks containing 5 ml of nutrient solution at the start of the experiment.

An analysis was performed of the effect of TRH (thyrotropin releasing hormone) (Thyrefact® Hoechst) and LH RH (luteinizing hormone-releasing hormone Op 81® Hoechst) on the extrusion of hormone into the organ culture. These solutions were added to the culture medium to a final concentration between 0 and 10^{-4} g/l. Pieces of tissue from GH and PRL producing and endocrinologically inactive tumours were investigated by this procedure. The incubation time was either 10 minutes or 4 hours whereafter the entire culture medium was renewed. Only one specimen was kept in each unit to allow correlation between morphological appearance and hormonal release.

III. *Morphological procedure*

The procedures for preparation of organ cultures for light and electron microscopy were described elsewhere by Anniko et al. (1979, in press).

RESULTS

I. *General Considerations*

Specimens were followed in vitro for 1–35 days. At termination of the culture period glu-

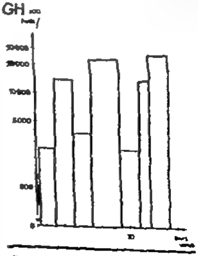

Fig. 2. Irregular GH production in vitro during 14 days (case no.).

Table I *Preoperative growth hormone (GH) and prolactin (PRL) levels in 10 patients with pituitary tumours later placed in organ culture*

Basal levels are means of two morning samples within 10 min before intravenous injection of 200 µg TRH. Peak values after TRH were in all instances reached 30 min after the injection. Normal range for GH 12–433 pmol/l, for PRL <25 µg/l

Patient no	Age	Sex	Endocrine insufficiencies	GH Basal (pmol/l)	GH Peak after TRH (pmol/l)	Prolactin Basal (µg/l)	Prolactin Peak after TRH (µg/l)
1	5	♀	–	350	1 850	10	86
2	60	♂	–	2 900	4 200	6	30
3	36	♂	LH FSH	950	1 470	15	52
4	2.	♀	LH FSH	4 700	6 800	–	4
5	34	♂	LH FSH	2 000	10 795	24	65
6	.2	♂	LH FSH	1 725	2 004	57	77
7	34	♀	LH FSH	Normal	No increment	166	160
8	24	♀	LH FSH	Normal	No increment	260	282
9	75	♀	LH FSH ACTH TSH	Normal	No increment	4	6.
10	64	♂	ACTH TSH	Normal	No increment	11	30

the tumour it was placed in Hanks balanced salt solution and under strict sterile conditions divided with a razor blade into tissue fragments not exceeding 1×1×1 mm. Thereafter the specimens were placed in Neumann & Tytell's serumless medium supplemented with 10% FCS (fetal calf serum) and 1% L glutamine. The incubation was performed in air atmosphere at +37°±0 2°C in maximum air humidity. The length of time from excising of the tumour to its incubation in the culture medium did not exceed 20 minutes.

Table II

Patient no	PRL In vivo	In vitro*
1	8	76
2	8	5
3	14	10
4	23	89
5	20	172
6	58	335
7	168	4 200
8	266	7 700
9	46	330
10	10	Not detectable

S-prolactin (PRL) values µg/l are means based on four morning samples.

PRL secretion in µg/l/48 h is the mean value of 6–10 specimens of the tumour.

Furthermore some specimens were also mechanically dispersed in Hanks BSS (balanced salt solution) and thereafter explanted to culture dishes containing Neuman & Tytell's serumless medium supplemented with 10% fetal calf serum and 1% L-glutamine.

The cultures were made either in culture dishes or in flat flasks containing only one specimen each. The nutrient solution was

Table III

Patient no	In vivo	In vitro	Clinical activity of acromegaly
4	4 100 (6)	3 700 (10)	+
	700 (10)	3 500 (10)	+
1	1 700 (8)	9 500 (6)	++
3	1 100 (9)	13 500 (10)	+++
5	600 (11)	35 500 (5)	+++
6	800 (8)	38 400 (10)	+++

Mean levels of fasting morning plasma growth hormone (GH) in pmol/l

* Mean level of in vitro GH secretion in pmol/l/48 h. Numbers within parentheses denote numbers of samples on which the means are based

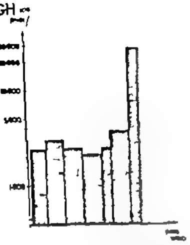

Fig. 5. The GH release could be stimulated by incubation with 10 g/l of TRH for 10 min increasing the hormone concentration severalfold from 4 285 pmol/l the basal level to 26 082 pmol/l after stimulation (case no 2). It should be noted that the culture medium was renewed completely both before and after the stimulation which means that the hormone levels in the probe from the stimulated tissue had to increase from zero level as the fresh medium to the stimulated value obtained at the analysis. The arrow indicates the start of the TRH incubation

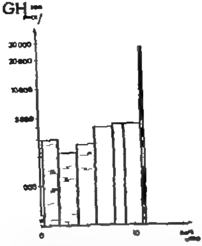

Fig. 6. Stimulation of GH release following incubation with 10^{-4} g/l of LH-RH during 24 hours. The hormone concentration increased from 680 pmol/l, the basal level to [illegible]490 pmol/l after stimulation (case no 2). The arrow indicates the start of the LH-RH incubation. Cf. Fig. 5

Ten different cultures containing mechanically dispersed pituitary cells were treated similarly to the organ culture specimens. The hormone production was initially kept at a high level but between the 2nd and 3rd weeks it decreased considerably reaching approximately 1/5 of the initial level. However hormone was present even after 35 days (Fig. 3)

2. Prolactin

Most specimens of PRL producing adenomas cultured were found to secrete high levels of PRL into the nutrient solution. Some organ cultures although from the same tumour secreted GH as well as PRL—the former however in very low concentrations as compared with PRL (GH 16—[illegible]1 pmol/l PRL 7000–8000 µg/l). This ratio of GH PRL tallied with the preoperative serum levels (patient 8). PRL-producing neoplasms were cultured for 16 days at maximum. During this time the specimens showed a fairly constant release into the medium (Fig. 4)

3. Pituitary tumour tissue lacking GH and PRL

The organ cultures of the adenoma from patient 10 with panhypopituitarism were morphologically preserved in the same way as GH and PRL producing tissues. However neither of these two hormones could be detected in the culture medium during the incubation of the specimens 14 days at maximum

4. Effect of TRH and LH RH on the release of tumour hormones

This study was restricted to GH-producing tissue.

TRH was a potent activator of GH release and a several fold increase in GH in the culture medium was obtained with a TRH concentration as low as 10^{-8} g/l incubated for 10 minutes (Fig. 5). The response was of the same magnitude when increasing the incubation time to 24 hours with the same concentration of TRH

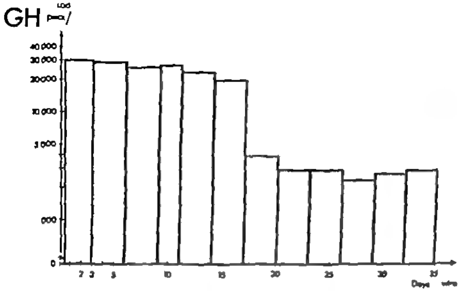

Fig 3 GH production in vitro during 35 days (case no. 6) from mechanically dispersed pituitary adenoma cells The endocrinologic activity decreased during the 2nd and 3rd weeks in culture but was thereafter maintained at a fairly constant level until the experiment ended after 35 days in vitro.

taraldehyde solution was added to the specimens after the culture medium had been removed for hormone analyses All tissue submitted to in vitro culture had pieces from the same adenoma fixed directly after surgical removal of the pituitary tumour to allow morphological comparison of tumour structure in vivo and after culture Each sample of the culture medium was analysed for GH and PRL.

II *Hormone Secretion in Vitro*

1 *Growth hormone*

In vitro there was a continuous and fairly constant release of hormone without any great fluctuations between the various measurements No apparent divergence in hormone production occurred between specimens cultured in culture dishes vis à vis flasks

The adenoma specimens cultured were grouped according to their secretory products Patients 1–3 had adenomas secreting GH with normal PRL production In specimens from patients 4–6 both PRL and GH secretion was noted (Tables II III) Adenomas with high serum levels of GH in vivo preserved a similar activity also after explantation to in vitro conditions even after extending the culture time to almost one month (Fig 1 and Table III) In order to analyse the extent to which hormone can be left in the culture following complete change of the medium the nutrient solution was repeatedly changed every 5 minutes. Already after the first rinse less than 1/10 of the initial hormone content remained indicating active hormone production/release into the culture medium

In a few instances great variability in hormone content of the nutrient solution occurred when changing the culture medium every 2 days (Fig. 2) A 3–4-fold difference could be noted between two adjacent probes taken at an interval of 2 days for instance

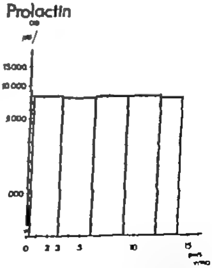

Fig 4 Prolactin secretion into the culture medium from pituitary adenoma tissue during 14 days in vitro (case no. [illegible]) The hormone production was maintained at a constant level without any sign of decrease during the investigation period

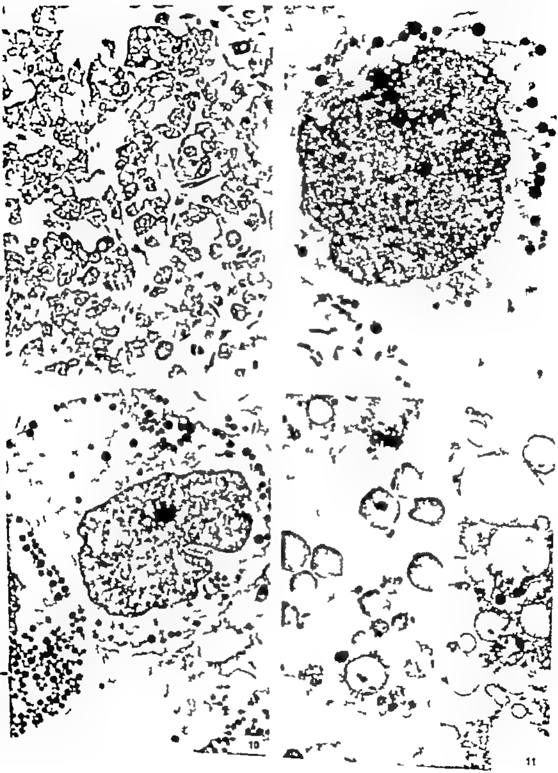
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Fig 7 Light microscopy (LM) When the adenoma tissue taken for organ culture was too large central necrosis often occurred within the first 1 days in vitro while the remaining pituitary cells remained morphologically preserved for a long period Prolactin producing tumour cultured in vitro for 14 days (case no 8) ×40

LH RH enhanced the GH release in vitro of GH producing adenoma cells (Fig 6) Stimulatory effects were obtained with a concentration of 10^{-7} g/l while incubation with 10^{-8} g/l of LH RH occasionally failed to increase the hormone content in the culture medium during short incubation periods as well as for 24 hours

Specimens from patient 10 with panhypopituitarism were incubated with TRH in concentrations from 10^{-8} to 10^{-9} g/l for 10 minutes and 24 hours respectively GH could be detected only in the culture media of two of nine specimens The concentrations obtained 23 and 41 pmol/l of GH correspond to serum levels noted in normal patients Thus the GH cells in the chromophobe adenoma maintained their differentiation as indicated by their unresponsiveness to TRH

III *Morphology*

All specimens subject to organ culture were sectioned for light microscopy and selected specimens for electron microscopy Good agreement existed between cell survival and hormone production i e a high hormone level in the culture medium was found only in specimens with preserved morphology (Figs 7 8)

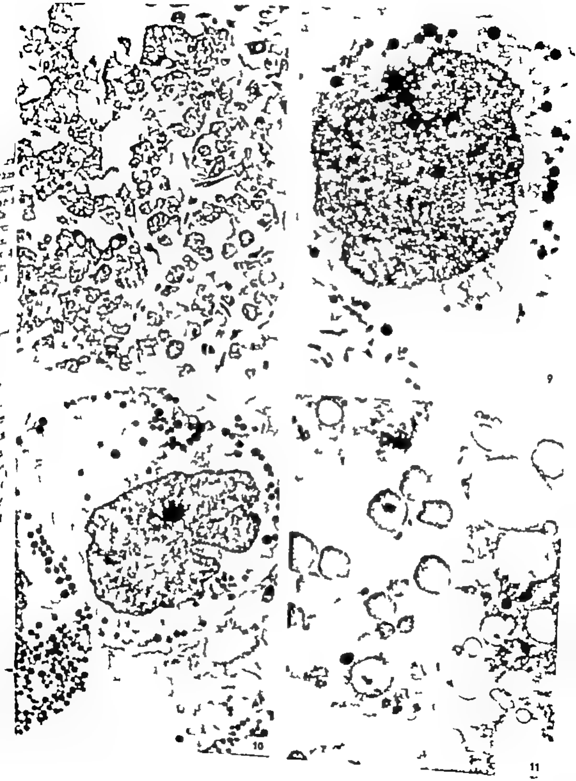
The ultrastructure of tumour cells cultured for various lengths of time appeared the same (Fig 9) as when fixed immediately after surgical removal (Fig 10) In specimens in which hormone release had been stimulated extrusion of granules was often observed from the cell surfaces Degenerating cells liberated hormone granules into the intercellular surroundings (Fig 11) by rupture of the cell membrane In the electron microscope hormone granules could sometimes be detected in endocrinologically inactive tissue (patient 10) but under basal conditions GH and PRL were undetectable in the medium by radioimmunoassay

Fig 8 LM Surviving GH-producing pituitary tumour cell after 28 days in vitro (case no 3) ×160

Fig 9 Electron micrograph (EM) Pituitary tumour cell from a GH-producing adenoma cultured in vitro during 1 days (case no 6) Preserved cell morphology with hormone granules peripherally located along the cellular membrane ×6000

Fig 10 EM Pituitary adenoma cell from the same tumour as in Fig. 3 Fixed immediately after surgical removal Secretory granules line the cell membrane ×6000

Fig 11 EM Liberated hormone granules following pituitary cell degeneration in vitro GH-producing adenoma cultured in vitro for 19 days (case no 1) ×40000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No mitoses were observed in sections from more than 200 different specimens at varying times after incubation

IV Correlation of clinical picture levels of PRL and GH and tumour morphology (Table III)

There was a positive correlation between serum PRL and PRL secretion in vitro (Table II) Pituitary adenomas diagnosed as mixed tumours secreting both GH and PRL (patients 5 and 6) maintained these characteristics also in vitro (Tables II III)

Tumour specimens from 3 patients (nos 3 5 and 6) produced exceedingly high amounts of GH which were not amply reflected in the preoperative plasma GH plasma samples (Table III) However the GH secretion rate in vitro correlated with the clinical activity of the disease (Table III) In all patients with acromegaly TRH infusion increased GH secretion in vivo (Table I) This pathological reactivity of the GH secreting adenoma cells was also demonstrated in all cases in vitro when TRH was added to the incubation media in concentrations of 10^{-6}–10^{-8} g/l as exemplified in Fig 5

DISCUSSION

In the present study the structural and functional integrity of the explanted tissue was maintained and was consequently suitable for the bioassay procedures in vitro

The biological quality of pituitary hormones obtained in vitro and in vivo is of special interest Binoux & Donnadieu (1975) reported that human GH from cell cultures of pituitary adenomas could not be distinguished from GH extracted from normal pituitaries with regard to physical chemical and immunological characteristics Furthermore Skyler et al (1977) reported that GH from adenoma tissue in vitro was indistinguishable from that obtained by extraction from normal pituitaries at autopsy However structural differences of PRL from the two sources were noted

In our experiments a high synthesis/release of GH and PRL occurred during at least one month and 2 weeks in culture respectively Peillon et al (1977) found in long-term cultures (7–15 days) of human somatotrophic tumours that PRL increased in 8/16 media samples while GH decreased during the same time in all instances

The fluctuations in hormone concentrations in some culture specimens (Fig 2, patient 4) may be explained by varying degrees of release of preformed hormone by intermittent synthesis and release in vitro of hormone by leakage from cells and/or by hormonal degradation within the medium It may be suggested that hormone production from adenomas in culture may be inhibited by the hormone itself If such a feedback mechanism is disturbed a great variability in hormone secretion may result A certain degree of steady state is obtained in specimens cultured in flasks when the medium is changed less frequently Therefore a certain hormone level is obtained within 1–2 days whereafter there may be only a minimal increase in hormone content in the medium

It is a well known fact that in patients with acromegaly GH release is stimulated by TRH and LH RH In the present study this pathological reactivity of the GH cells was demonstrable in vitro too Similar results have been reported by Goodyer et al (1977) concerning human fetal pituitary cells in culture (LH RH used 10^{-6}–10^{-8} M TRH 10^{-8} M)

In the studies of Tixier Vidal et al (1970, 1975) on rat pituitaries in organ culture it was shown that after incubation with LH RH for 12 days the LH cells enlarged became irregular in shape the secretory granules diminished and the Golgi zone became considerably enlarged There is a lack of corresponding data on human material However the concentrations used in our experiments were considerably lower as were the incubation periods This may explain the lack of specific morphological changes after stimulation with TRH and/or LH RH in the present work

To sum up it is possible to reserve human

pituitary tumours in organ culture and to maintain the initial morphology and level of hormone production. Furthermore the adenomas retain their capacity to respond with hormone release in the presence of substances which increase the secretion in vivo. Our findings indicate that in all patients with hypersecretion of GH and/or PRL this was due to an autonomous pituitary adenoma. All specimens from the nine pituitaries maintained increased hormone production even when extrapituitary influences, such as from the hypothalamus, were excluded. The secretory activity of the somatotrophic adenomas in vitro was not regularly reflected in plasma GH but was illustrated by the signs and symptoms of activity of the disease, i.e. headache, sweating, soft tissue swelling and hypertension (Table III). Repeated samplings of serum PRL, on the other hand, offered a good estimate of the hormonal activity of the prolactinomas (Table II).

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ZUSAMMENFASSUNG

Menschliche pituitäre Adenome behalten in der Organkultur ihre Hormonsynthese und ihr Hormonfreilassen. In dieser Weise sind zuwachshormonproduzierende Adenome 35 Tage und Prolaktinome 14 Tage mit beibehaltener Hypersekretion (> 30 000 pmol/l/48 h Zuwachshormon und > 1 000 μg/l/48 h Prolaktin) kultiviert worden. In diesen Proben wurde beibehaltene Morphologie/Ultrastruktur dokumentiert. Nach vollständiger wiederholter Erneuerung der Nahrungslösung blieb nach der ersten Erneuerung weniger als 1/10 des ursprünglichen Hormoninhalts übrig. Aber eine aktive Hormonproduktion/ein aktives Hormonfreilassen in das Kultivierungsmedium als die Hormonkonzentration wie zu ihren früheren Niveaus steigend andeutet.

Das zellenproduzierende Zuwachshormon konnte durch TRH und LH RH in den Konzentrationen von respektiv 30 und 10 μg/l stimuliert werden, dieselbe pathologische Reaktivität wie in vivo andeutend. Wie die Serumprolaktinniveaus abgegeben, entsprach die Prolaktinsynthese in vitro derselben in vivo. Die Zuwachshormonsynthese in vitro entsprach der klinischen Aktivität der Krankheit, jedoch nicht den Plasmahormonniveaus in Patienten mit Akromegalie.

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Mats Anniko M.D.
Department of Otolaryngology
Karolinska Sjukhuset
S-104 01 Stockholm Sweden

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NODULAR FASCIITIS IN THE HEAD AND NECK

A Clinicopathological Study of 18 Cases

I Dahl and J Jarlstedt

From the Departments of Pathology University of Goteborg and Central Hospital Vanersborg and the Department of Otolaryngology University of Göteborg Sweden

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Abstract Nodular fasciitis a benign pseudosarcomatous proliferative lesion of the soft tissue is frequently misinterpreted as sarcoma both clinically and microscopically A series of 18 cases of nodular fasciitis in the head and neck region is presented The clinical observations and the light microscopy are described Ten out of the 18 cases were situated deep in the soft tissue six were located close to the mandible and another 6 along the sternocleidomastoid muscle seeming to support the view that nodular fasciitis may be ascribed to a reparative response to local mechanical events caused by injury The diagnosis and differential diagnosis in relation to other benign and malignant tumours occurring in the region are discussed The importance of otolaryngologists being aware of the existence of this entity in this area of the body is stressed Follow-up information for 12 of the patients confirmed a perfectly benign clinical course and simple excision as tissue-sparing as possible is therefore the treatment of choice

Nodular fasciitis originally described by Konwaler et al (1955) is an entirely benign and the most common pseudosarcomatous lesion of the soft tissue (Dahl & Angervall 1977) The lesion may easily be misinterpreted as sarcoma on clinical grounds owing to its often rapid growth without signs of concurrent infection and light microscopically owing to high cellularity and mitotic activity and a frequent indistinct delineation An erroneous diagnosis of sarcoma has been recorded in up to 50% of retrospectively collected cases (Price et al 1961 Stout 1961 Soule & Minn 1962 Allen 1972 Dahl et al 1972)

About 15% of all cases of nodular fasciitis involve the head and neck (Enzinger 1965 Kleinstiver & Rodrigues 1968 Dahl et al 1972 Allen 1972) Case reports of nodular fasciitis in the head and neck have appeared in oral and dental journals (Smith 1967 Henry et al 1969 Rakower 1972 Lumermann et al 1972 *Solomon et al 1974* Larsson & Svartz 1976) but no series of cases of nodular fasciitis in this region has previously been *analysed*

This paper presents a clinicopathological study of 18 cases of nodular fasciitis in the head and neck Owing to erroneous diagnosis of malignant tumour 4 of the patients were subjected to extensive surgery

PATIENTS AND METHODS

Four out of the 18 cases were obtained from a Swedish series comprising some 800 cases of

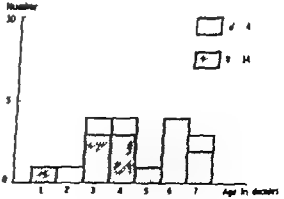

Fig 1 The sex and age distribution of 18 cases of nodular fasciitis in the head and neck

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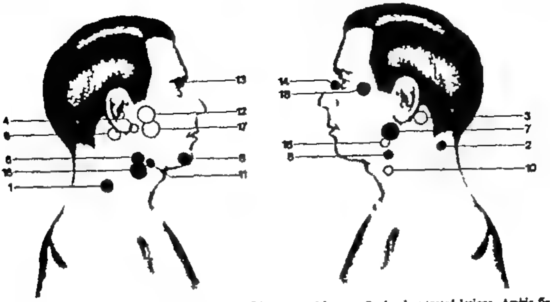


1 Anatomical location of 18 cases of nodular fasciitis in the head and neck ○ Superficially (subcutaneously) situated lesions ● deeply situated lesions Arabic figures case numbers

malignant soft tissue tumours reported to the Swedish Cancer Registry during a 6-year period (1958–1963) Seven cases were selected after reviewing various soft tissue tumours recorded at the Departments of Pathology University of Göteborg and Central Hospital Vanersborg Three tumours were sent for consultation (University of Goteborg) from other pathological laboratories The additional 4 cases seen in the period from 1966 and onwards were diagnosed as nodular fasciitis.

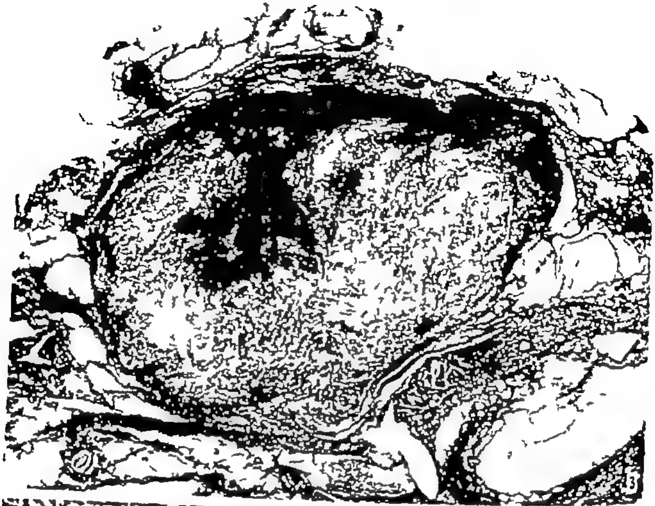
Histological methods Sections of tissue routinely stained with haematoxylin and eosin and/or the haematoxylin–van Gieson method were available for review in all cases. In 1 case 5-micron thick sections of paraffin-embedded tissue were stained by the Gordon Sweet method for reticulin fibres The Prussian-blue reaction for iron-containing pigments and the periodic acid Schiff reaction (McManus) with or without prior treatment of the section with diastase (Merck) was performed Alcian blue (Chroma-Gesellschaft) stain for the examination of mucosubstances was used at pH .5 and 0.5 with and without prior treatment of the sections with testicular hyaluronidase (hyaluronidase from bovine testes type IV Sigma)

RESULTS

The sex and age distribution of the patients are shown in Fig. 1 The youngest patient was 6 years old and the oldest 68 years The median age was 37.5 years

The anatomical location of the tumours is shown in Fig. _ Five lesions were superficially situated entirely or almost entirely in the subcutaneous tissue and 10 lesions in the deep fascia or contiguous striated muscle Two lesions (cases 1 and 17) were situated in the superficial part of the parotid gland and one lesion deep in the posterior-lateral wall of the hypopharynx (case 16)

The growth rate of the tumours was known in all cases being less than 1 month in 4 between 1 and ? months in 9 and more than 3 months in 4 One of these 4 lesions had been slowly growing for 8 months Mild pain and tenderness were experienced by 5 patients A



definite history of trauma before the appearance of the tumour was obtained from 1 patient

Pathology The maximum diameter of the lesion was less than 1 cm in 8 cases between 1 and 2 cm in 6 cases and greater than 2 cm in 4 cases The largest lesion was approximately 4 cm in diameter

Grossly the lesion was well demarcated nodular or almost nodular in 11 cases (Fig. 3) and more or less ill-defined growing more diffusely extending along fascial planes and sep-radiating into adjacent tissue in 7 cases

The cut surface was firm grey to greyish white in all cases soft myxoid or gelatinous in cases and tinged with yellow and pink in 1

Microscopically all the lesions showed proliferation of fibroblasts of varying shape Proliferating thin elongated s-formed or wavy cells mixed with plumper cytoplasmic elongated cells were seen in all cases (Fig 4) The s-formed or wavy cells showed thin elongated nuclei with pointed ends and separated slit-like spaces or small clefts (Fig 5) The nuclei of the plump cytoplasmic cells were oval with delicate chromatin with small chromatin clumps and nucleoli (Fig 5) These cells were arranged more compactly and focally in bundles (Fig 5) Large mono- (Fig. 9) and multinucleated cells were a prominent finding in 10 lesions All lesions were rich in capillaries either irregularly branching haphazardly arranged or arranged in a radial pattern with extravasated erythrocytes (Figs 6 and 7) Lymphocytes plasma cells, histiocytes and mast cells in moderate amounts were diffusely distributed within the lesions or arranged in small groups. Signs of blood resorption with deposition of haemosiderin in small quantities were seen occasionally The amount and type of interstitial ground substance varied within and between the lesions It was predominantly collageneous in 12 lesions with collagen and reticulin fibres arranged in parallel with the proliferating cells Denser haphazardly arranged bundles were prominent in 5 lesions (Fig 8) In 6 lesions the interstitial ground substance was abundant myxoid (Fig 9) The myxoid matrix in all lesions showed alcianophilia at pH 2.5 as well as at pH 0.5 and testicular hyaluronidase with digest hyaluronic acid as well as chondroitin 4- and 6-sulphates (Zugibe 1970) reduced the alcianophilia at pH 2.5 and abolished it at pH 0.5

Mitoses were evident in all lesions particularly in more cellular areas In 5 lesions the mitotic frequency was approximately one per high-power field (×400) Atypical mitotic figures were not observed

Fig. 3. Nodular or almost nodular well-demarcated form of nodular fasciitis situated in the subcutaneous tissue. Note the connection with connective tissue septa. Case Haematoxylin–van Gieson ×6

Fig. 4. Cellular area in nodular fasciitis showing mixture of proliferating thin elongated s-formed or wavy cells and plump cytoplasmic elongated cells. Haematoxylin–van Gieson ×100

Fig. 5. Plump, cytoplasmic cells with oval nuclei arranged compactly or in bundles (upper half) and s-formed or wavy cells with thin elongated nuclei separating small clefts or slit-like spaces (lower half). Haematoxylin–van Gieson, ×60

PRIMARY DIAGNOSES

The primary diagnosis in 4 of the 11 retrospectively collected cases was fibrosarcoma and in one case neurofibrosarcoma In a further 2 cases malignancy was suspected one as fibrosarcoma and one as neurofibrosarcoma One case was initially diagnosed as a cellular neurinoma with a high risk of recurrence In 3 cases the initial diagnoses were fibromyxoma, neurofibroma and leiomyoma The remaining 7 cases seen in the period from 1966 and onwards were diagnosed as nodular fasciitis

TREATMENT AND FOLLOW UP

Local excision of the lesion was performed in all cases. Because of an initial diagnosis of sarcoma, wide re-excision of the scar area was performed in 4 patients In one of these patients 2 more re-excisions as well as regional

lymph node dissection were performed. In another patient, with the tumour in the parotid gland, resection of the superficial as well as the deep portion was performed and the facial nerve was sacrificed because an initial frozen sectioned biopsy suggested malignancy. Successful transplantation of the great auricular nerve was performed peroperatively in this case.

Follow-up information was available for 17 patients and covered a period ranging from 7 months to 16 years, with a median of 6.5 years. One patient died of an intercurrent disease. The remaining patients are alive and well without signs of recurrence or metastasis.

DISCUSSION

All lesions in this series fulfilled the microscopic criteria for a diagnosis of nodular fasciitis. Histologically, nodular fasciitis has been divided into different forms (Price et al. 1961, Enzinger 1965, Allen 1972), almost all of which were represented in our series. However, there can be a great variation within the lesions as well as between them, making classification difficult. There seems to be no correlation between the histological type and the clinical course (Price et al. 1961, Hutter et al. 1962, Font & Zimmerman, 1966, Mehregan 1966, Meister et al. 1978*a*). Therefore subclassification is of less importance; more important is the recognition of the whole spectrum of these lesions.

Clinically, the lesion mostly appears as a rather rapidly growing tumour, but sometimes the course is more prolonged, extending over several months as in 4 cases in this series. Almost all the lesions in the series were small, but they may, as in 3 cases in the series, be as large as 3–4 cm in maximum diameter.

The age distribution of the patients in this series of nodular fasciitis in the head and neck did not differ from that in large series of nodular fasciitis on the trunk and extremities (Dahl). The female predominance is probably due to the relatively small number of patients. In large series nodular fasciitis occurs with almost equal frequency in the two sexes (Stout 1961, Soule & Minn 1962, Enzinger 1965, Allen 1972, Meister et al. 1978*b*).

Nodular fasciitis is in most cases subcutaneously situated but affects deep muscle fascia and/or adjacent striated muscle in about 15–20% of cases in large series (Price et al. 1961, Enzinger 1965, Allen, 1972, Dahl, Meister et al. 1978). It is interesting to note that 10 out of the 18 lesions were situated deep in the muscle fascia and/or adjacent striated muscle. Six of the lesions were located in the soft tissue near the mandible and another 8 lesions along the sternocleido mastoid muscle, areas which probably are predisposed to trauma during mastication and movements of the head. Nodular fasciitis can possibly be ascribed to a reparative response to local mechanical events caused by injury (Solomon et al. 1974, Dahl & Angervall 1977).

Areas of special interest to otolaryngologists where occasional cases of nodular fasciitis have been described are the trachea and esophagus (Stout, 1961, Kleinstiver & Rodrigues 1968, Allen 1972) and, as in this series, the parotid gland and hypopharynx.

The differential diagnostic problems that may occur in connection with nodular fasciitis have been discussed in larger series (Price et al. 1961, Allen 1972, Dahl *et al.* 1977) and are further illustrated by the fact that a diagnosis of neurofibro- or fibrosarcoma or probable neurofibro- or fibrosarcoma was proposed for 7 tumours in this series. These dia-

Fig. 6. Myxoid area in nodular fasciitis showing proliferating capillaries radiating into the adjacent subcutaneous tissue. Haematoxylin and eosin, ×40.

Fig. 7. Proliferating capillaries, either irregularly branching or arranged in a radial pattern with extravasated erythrocytes. Small amounts of inflammatory cells are diffusely distributed. Haematoxylin and eosin, ×100.

Fig. 8. Collagen-rich area in nodular fasciitis. Dense haphazardly arranged bundles of collagen mixed with proliferating cells. Haematoxylin-van Gieson, ×100.

Fig. 9. Myxoid area in nodular fasciitis with haphazardly distributed proliferating cells in an abundant myxoid matrix. Note large mononucleate cell in the centre. Haematoxylin-van Gieson, ×100.

gnoses were obviously based on conventional histological criteria for malignancy such as high cellularity cellular and nuclear polymorphism high frequency of mitoses and infiltrative growth These criteria are not valid for nodular fasciitis

The appearance of the lesion at low magnification under the light microscope is often conclusive in distinguishing it from sarcoma Nodular fasciitis extends along fascial planes and subcutaneous septa radiating into the fat lobules or around and into bundles of muscle fibres in adjacent striated muscle Furthermore the compact appearance of intertwining bundles of spindle shaped cells and anaplasia of the proliferating cells in sarcoma are never seen in nodular fasciitis

Neurilemmoma (schwannoma) a diagnosis proposed for one case in the series are frequent in the head and neck (Ehrlich & Martin 1943 Das Gupta et al 1969) Neurilemmoma are characterized by the mixture of Antoni type A and type B tissues The cells of the Antoni type A tissues are plumper with a tendency to regimentation of the nuclei in twisted rows or palisades (Enzinger et al 1969) Mitoses areas containing proliferating capillaries and inflammatory cells are frequent findings in nodular fasciitis and are not seen in neurilemmoma

Aggressive fibrous lesions of the oral cavity may be difficult to distinguish from nodular fasciitis (Larsson & Björlin 1976) Aggressive fibrous lesions frequently show bone destruction which was not evident in any case in this series of cases of nodular fasciitis and microscopically these lesions exhibit features of fibromatosis with a uniform growth pattern abundance of collagen and paucity of mitoses (Enzinger et al 1969)

The clinical course in patients with nodular fasciitis is as in this series perfectly benign Recurrences may occur (Stout 1961 Kleinstiver & Rodrigues 1968 Allen 1972 Meister et al 1978) but spontaneous regression of the lesion is perhaps more common (Hutter et al 1962 Soule & Minn 1962 Mehregan 1966 Kleinstiver & Rodrigues 1968 Kovac et al 1970 Dahl et al 1972) Simple local excision as tissue-sparing as possible without risking the function of the area involved is considered the treatment of choice Aspiration biopsy may be of value in the diagnosis (Dahl & Åkerman) and in planning the operation.

ZUSAMMENFASSUNG

Die noduläre Fasciitis eine benigne pseudosarkomatöse proliferative Erkrankung der Weichteile wird häufig sowohl klinisch als auch bei der histologischen Untersuchung als Sarkom fehlinterpretiert. Im vorliegenden Bericht wird eine Serie von 18 Fällen von nodulärer Fasciitis der Kopf und Halsregion vorgestellt. Klinische Befunde und Histologie werden beschrieben Von der Gesamtzahl von 18 Fällen fanden sich zehn im tiefen Weichteilgewebe sechs in den Unterkieferweichteilen im Mandibulabereich weitere sechs im Verlauf des M sternocleidomastoideus Diese Lokalisationen scheinen die Vermutung zu unterstützen daß die noduläre Fasciitis die reparative Antwort auf durch Verletzung hervorgerufene mechanische Schäden darstellt. Diagnose und Differentialdiagnose gegenüber anderen in diesem Gebiet vorkommenden benignen und malignen Tumoren werden diskutiert Die Wichtigkeit für Otolaryngologen an das Vorkommen der Erkrankung in dieser Körperregion zu denken wird hervorgehoben Spätere Kontrollen bei zwölf der Patienten zeigten einen durchweg benignen Verlauf daher ist die einfache gewebeschonende Exzision die Therapie der Wahl

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Ingvar Dahl M.D
Central Hospital Fack
S-462 01 Vänersborg
Sweden

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ULTRASTRUCTURE OF SYNAPSES IN THE LATERAL LINE CANAL ORGAN

G Benshalom and Å Flock[a]

From the King Gustaf V Research Institute Stockholm, Sweden

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Abstract The ultrastructure of afferent and efferent synapses on hair cells in the lateral line canal organ of the fish *Lota lota* was studied utilizing various fixation and staining techniques New information was obtained about membrane-associated material such as the presynaptic body and postsynaptic densities, by examining glutaraldehyde-fixed material not subjected to osmication Contrast was instead obtained either by section staining with uranyl acetate and lead citrate or by block impregnation with phosphotungstic acid (PTA). PTA-staining enhances the postsynaptic membrane of both the afferent and efferent synapses. It also stains the presynaptic dense projections at the efferent synapse and in a differential fashion, the afferent synaptic body Section staining reveals substructure in the feet of the afferent synaptic body faintly seen but masked in osmicated tissue

The purpose of this work was to investigate in detail the structure of synapses of sensory hair cells and to determine the relationship of associated material to the component membranes in the synapse In particular we were interested in the synaptic body and electron-dense structure found presynaptically at the afferent synapse schematically presented in Illustration 1 At this site neurochemical synaptic transmission is believed to occur The transmitter release process probably involves events in which not only synaptic membranes but also these membrane associated materials participate (Szentágothai 1970; Bloom et al 1970 Pfenninger 1973) This material which has been commonly observed upon fixation with osmium tetroxide has been studied in the present work by omitting the postfixation with osmium after glutaraldehyde fixation and by staining with phosphotungstic acid.

Hair cells of the lateral line organ possess two types of synapse of quite distinct structure and function located side by side on the same sensory cell The physiology of both synapses is well known from previous investigations (Flock & Russell 1976) One type of synapse is afferent and excites the sensory nerve endings of the lateral line nerve, the other is efferent and inhibitory (Illustrations 1 and 2) (Russell, 1968 1971*a* Russell & Roberts 1972 Flock & Russell 1973*a* 1973*b* Hashimoto et al 1970) At the afferent synapse vesicles are found in the hair cell surrounding the synaptic body while at the efferent synapse vesicles are present in the nerve terminal (Hama, 1965 Hama & Saito 1977 Flock, 1965 Jande 1966 Roberts & Ryan 1971 Shelton, 1971 Jorgensen & Flock 1973 Nakajima & Wang 1974) Whereas the efferent transmitter is likely to be acetylcholine, that of the afferent synapse is unknown (Russell 1971*b* Flock & Russell 1976 Flock & Lam 1974)

Because the lateral line organs have the same embryological origin as the sense organs of the inner ear to which they are similar structurally and functionally what is learned here for lateral line organs should in general apply also to inner ear sense organs (Bergeijk 1967)

Present address. Section on Functional Neuroanatomy NINCDS, National Institute of Health, Bethesda, MD 20205 Bldg 36 Rm 3B26, USA.

[a] Present address. Department of Physiology II, Karolinska Institutet, S-10401 Stockholm 60 Sweden

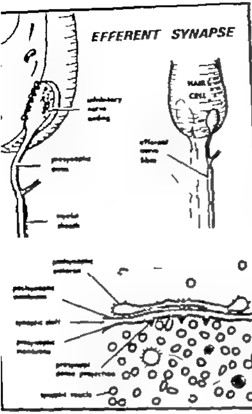

Illustration 2 Schematic representation of the efferent Synapse on hair cells and its general structure

discerned between preparations directly fixed with osmium tetroxide and those prefixed with glutaraldehyde followed by postfixation with osmium.

The afferent synapse (Figs. 1–1)

Afferent synapses in organs fixed or postfixed with osmium tetroxide and double stained by uranyl-acetate and lead-citrate (Figs 1–5) show the following characteristics The post synaptic membrane is thicker than the rest of the nerve membrane due to its post synaptic density This electron-dense web becomes gradually dispersed towards the inner face of the nerve until it disappears in the cytoplasm.

The presynaptic membrane which looks thinner than the postsynaptic membrane has the same width as the rest of the hair cell membrane and shows no increased density

The synaptic gap (synaptic cleft) between the pre and postsynaptic membranes appears more electron-dense than the rest of the extracellular space between the hair cell and the nerve cell Within the synaptic gap dark filaments are often seen to bridge the pre- and postsynaptic membranes (Figs. 3 4) These filaments may be responsible for the constant distance that is kept between these two membranes throughout the synaptic area.

The pre- and postsynaptic membranes run in parallel They take a wavy course when viewed in one plane of sectioning (Figs. 2 and 5) while in a perpendicular plane they exhibit a gently curved shape (Figs. 1 3 and 4) Thus the presynaptic area which faces the hair cell cytoplasm has corrugated impressions which form elongated grooves These are seen in the freeze-etch replica of Fig 6 showing a patch of presynaptic membrane seen from the inside of the hair cell When the presynaptic membrane is viewed from the outside of the hair cell ridges studded with particles are seen (Fig 7)

Inside the hair cell on the presynaptic side of the synapse a special organelle is present which is known as the synaptic body' The synaptic body has two subunits The synaptic body's feet' are elongated structures each of which fits closely into a groove of the pre synaptic membrane In cross section they look like round bodies that have about the same diameter as the synaptic vesicles (Fig. 5) and in longitudinal sections they are suspected to exhibit a tubular substructure (Figs 4 10) As many as 14 feet have been counted in association with a single synaptic body The second subunit of the synaptic body the main body mass lies deeper in the hair cell cytoplasm and is connected to the feet by thin dense filaments (arrows, Figs. 3 5) The main mass of the synaptic body varies in shape and size It can extend beyond the area of the feet and has a protuberance pointing towards the hair cell cytoplasm (Figs 1 and 2) Thus if a

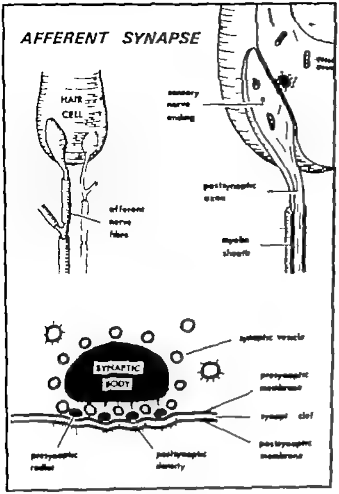

Illustration 1. Schematic drawing of the general structure of the afferent synapse on lateral line hair cells.

MATERIALS AND METHODS

The work was done on the lateral line canal organs of the teleost fish (burbot) *Lota lota* The supra temporal organs of adult animals were dissected as previously described (Flock 1965) and were fixed for 2 hours with one of the following fixatives

(1) 1% Osmium tetroxide buffered to pH 7 4 with veronal acetate (Rhodin 1954) This fixation was done either immediately after dissection or after immersing the organs for 2 hours in Cortland's physiological solution for fresh water fish (Wolf 1963) at 4°C

(2) 3% glutaraldehyde in 0 133 M phosphate buffer pH 7 4 (Sabatini et al 1963) or in 0 08% s-collidine buffer with 5% sucrose pH 7 4 Part of the glutaraldehyde fixed organs were postfixed for 2 hours with 1% osmium tetroxide and the rest were left without postfixation

Dehydration was carried out in alcohol At the end of the dehydration process some of the glutaraldehyde fixed organs were block stained in 1% phosphotungstic acid in absolute ethanol E PTA (Bloom & Aghajanian 1966) while the others were left without any block staining

After the specimens were embedded in Epon (Luft 1961) thin sections (grey silver) of the osmium tetroxide treated specimens and slightly thicker sections (yellow-buff) of the glutaraldehyde fixed organs were cut on a LKB Ultrotome Half of the E PTA block stained sections were left for electron microscope observations without any additional staining All the other sections of the various treatments described above including the remaining half of the E PTA block staining sections were double stained with uranyl acetate and lead citrate (Reynolds 1963)

Electron microscopy was performed using a Siemens Elmiskop I

For freeze fracturing lateral line organs were fixed 6–12 hours in 2 5% glutaraldehyde in sodium-cacodylate buffer (0 1 M pH 7 4). They were immersed for 30 min in 30% glycerol mounted on gold specimen holders and quenched in liquid nitrogen brought to freezing temperature by evacuation Fracturing was done with a double replica holder in a Baltzer 360 M freeze-etching apparatus. After etching for 60 sec replication was done with an electron gun to a thickness of 200 Å as determined by a quartz crystal monitor The replicas were examined in a Zeiss EM 9 electron microscope

OBSERVATIONS

Illustrations 1 and 2 are schematic drawings of the afferent and efferent synapses of osmium tetroxide fixed double stained preparations which summarize the observations presented in the micrographs of figures 1 to 5 and 13 to 17 No major structural differences could be

much smaller than the width of the synaptic cleft This narrow intracellular gap is also kept constant and the two membranes on either side of it are connected to each other by electron-dense material The membrane of the cisterna facing the inner part of the hair cell has a more irregular shape Electron-dense ribosome-like granules commonly in groups of three are located on the cytoplasmic membrane of the cisterna, mainly (but not only) opposite presynaptic dense projections

In glutaraldehyde fixed double stained preparations, the efferent nerve membrane is faintly outlined in an irregular discrete manner (Fig 18) However the synaptic regions are more prominent (Figs 18–20) Postsynaptic and presynaptic membranes appear to be composed of two units (Fig 20) in a way reminiscent of the separation of the postsynaptic membrane of the afferent synapse

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Fig 8 9 Two successive cross-sections of an afferent synaptic area transversely sectioned through synaptic body main mass (Fig 8) and its feet (Fig 9). Glutaraldehyde-fixed tissue, double-stained with uranyl acetate and lead citrate 77 900

Fig 10 Higher magnification of the centre of figure 9 The synaptic body feet possess denser sheet around themselves (arrow) (See also Hollinger 1964 Fig 12.) 100 000

Fig 11 Higher magnification of the afferent synapse seen in Fig 1 Note the denser sheet around the synaptic body feet the separation of the postsynaptic membrane (thin and thick arrow point to the outer and inner layers, respectively) and the smoother contour of the membranes as compared with their shape in Fig 2 75 000

Fig 12 Survey picture of the bottom of hair cell in glutaraldehyde-fixed double-stained tissue A afferent nerve ending is tightly attached to the hair cell in the synaptic region The swelling extracellular space around the synaptic junction is commonly seen under this fixation condition, and ascribed to the already known high osmolarity of the fixative 45 000

Fig 13 Survey picture of the bottom of hair cell Two efferent nerve endings are seen The left one possesses an efferent synapse, shown under higher magnification in Fig 15 The arrow points to two synaptic body-like particles Osmium tetroxide fixed, double-stained 11 500

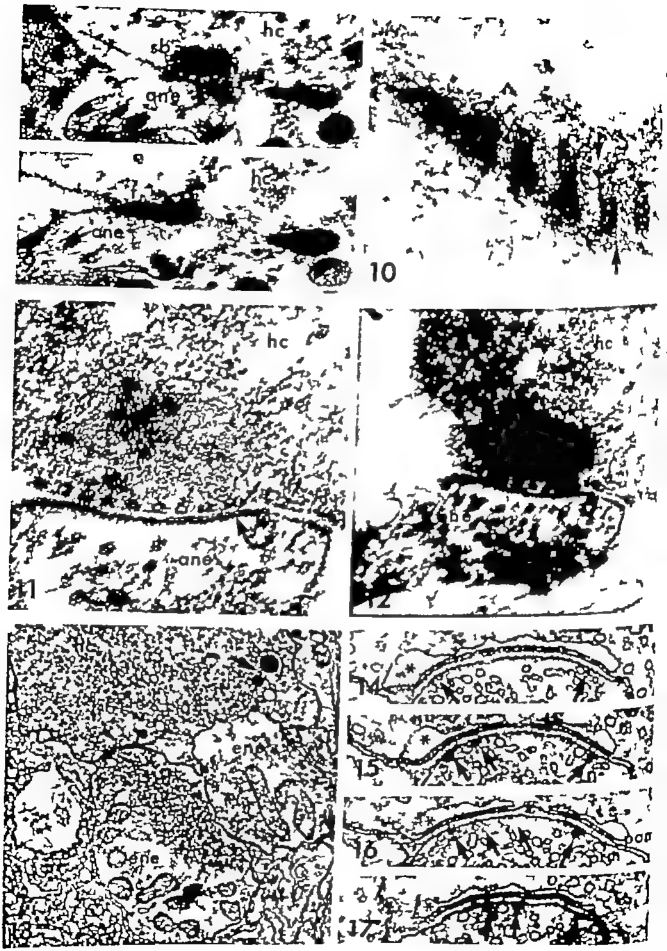
Fig 14-17 Serial sections of the efferent synapse seen in Fig 13 The postsynaptic cisternae (thin arrows) ramify into two separate tubes (Figs 15 16) which combine to form one tube on each side (Figs 14 17) Note the random array of the presynaptic dense projections (arrows) 7000

by an identical preparation procedure (Fig 11) Presynaptic dense projections are clearly observed while synaptic vesicles are not seen as membranous organelles The postsynaptic cisterna tends to swell. Only its section of membrane opposite to the postsynaptic membrane is prominent while the section of membrane facing the hair cell cytoplasma is invisible although its position is sometimes outlined by the ribosome like granules on its surface

E PTA block staining of afferent and efferent synapses (Figs 21–24)

In glutaraldehyde-fixed E PTA impregnated organs the afferent and efferent synapses are stained in a specific way (Figs 21–24) In the afferent synapse the two components of the postsynaptic membrane which have been observed in glutaraldehyde fixed, double-stained preparations are remarkably well stained by the E PTA while the presynaptic body is selectively stained Its feet as well as the outer layer of its main mass appear noticeably electron-dense while the core of the synaptic body is not stained at all and appears much brighter than the hair cell's cytoplasm (Fig. 21) *Double-staining of sections of this preparation* with uranyl acetate and lead citrate stains the whole synaptic body including the core of the E PTA-treated synaptic body (Fig 22) which appears this time as electron-dense as its outer E PTA-stained layer

In the efferent synapse the presynaptic projections and the postsynaptic membrane are stained by E PTA while the presynaptic membrane is faintly recognized, if at all (Fig. 23) After double-staining these preparations with uranyl acetate and lead citrate (Fig. 24) the efferent synapses take on an appearance similar to glutaraldehyde fixed, double-stained preparations which were not treated with E PTA (compare Fig. 18 with Fig. 4) As mentioned above in glutaraldehyde-fixed organs which were not fixed with osmium but stained with either E PTA or with uranyl and lead the membranes of synaptic vesicles are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not seen. However, the cytoplasm in the basal part of the hair cell contains particles suspected of being the shadows of synaptic vesicles (Figs. 11, 12, 18, 20, 21–24).

No noticeable post-mortem effects were found in the tissue of organs immersed for 3 hours in physiological solution before fixation. The synapses of both types looked the same as in organs fixed *in situ*. This observation is in agreement with the data reported previously that isolated lateral line organs can produce physiological responses for several hours (Flock, 1965).

DISCUSSION

Although much has been learned about synaptic physiology and ultrastructure in auditory and vestibular organs in recent years, much remains to be explored. The identity of the afferent excitatory transmitter is still unknown and the functional role of efferent nerve fibres in the hearing organ is not settled. Another intriguing question is that of the significance of the presynaptic dense body at the afferent synapse. An important breakthrough has come with application of the freeze fracture technique to the study of inner ear synapses (Jahnke, 1976; Gulley & Reese, 1977; Bagger-Sjöbäck & Flock, 1977). In particular the elegant work of Gulley & Reese (1977) has demonstrated differences in the structure of membranes and associated material at afferent and efferent endings in the organ of Corti.

We have attempted to distinguish ultrastructural characteristics of the two types of synapse by the use of differential fixation and staining techniques. We would first like here to discuss the results obtained by these techniques and then turn to the possible significance of the synaptic body.

Two fixatives were used: osmium tetroxide, which is the most common fixative, and glutaraldehyde which was used here, and in a previous work, without any post-fixation (Ben-Shalom, 1979). Without exposure to osmium tetroxide, cell membranes were invisible unless stained. Staining of the non-osmicated preparation was done either with ethanolic PTA block staining, which is known as a selective staining for synaptic complexes (Bloom & Aghajanian, 1966, 1968), or by double staining of sections with uranyl acetate and lead citrate. The E-PTA selectively stains substances that are rich in non-specific basic amino acids and leaves the rest of the cell's components unstained, while section staining reveals more details of components and helps to relate E-PTA-stained material to associated structures.

Comparing tissue fixed with osmium tetroxide or glutaraldehyde, differences in the quality of the tissue preservation appear. The wavy contour of the membranes at the afferent synapse is not preserved by glutaraldehyde alone if the tissue is embedded and sectioned. However, waviness is preserved in freeze-fractured

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the synaptic cleft and in the gap between the hair cell membrane and the postsynaptic cisterna (arrow). Arrow points to a single presynaptic dense projection. ×91 000.

Fig. 20. Efferent synapse in glutaraldehyde-fixed tissue stained with the double-staining technique. Note the separation of the postsynaptic and presynaptic membranes into two leaflets, as compared with their structure in Fig. 19. The section of membrane of the postsynaptic cisterna facing the hair cell cytoplasm is invisible, while that section facing the hair cell membrane is intensely stained (arrow). ×92 000.

Fig. 21. Afferent synapses in glutaraldehyde-fixed tissue block stained with E-PTA. Note the special appearance of the synaptic body and the postsynaptic membrane. Nerve mitochondria are also intensely stained. Thin and thick arrows point respectively to the outer and inner layers of the postsynaptic membrane (cf. Fig. 11). ×50 000.

Fig. 22. Afferent synapse. Glutaraldehyde-fixed, block stained with E-PTA followed by double staining of the sections with uranyl acetate and lead citrate. The synaptic body feet are longitudinally sectioned. ×50 000.

Fig. 23. Efferent nerve ending with efferent synapse (circumscribed between two arrows) at the bottom of a hair cell in glutaraldehyde-fixed tissue, block-stained with E-PTA. The presynaptic dense projections are seen opposite to the remarkably stained postsynaptic membrane. ×22 000.

Fig. 24. Efferent synapse. Glutaraldehyde-fixed, block stained with E-PTA, followed by double staining of the sections with uranyl acetate and lead citrate. Non-synaptic membranes are indiscernible even after application of both staining procedures. ×46 000.

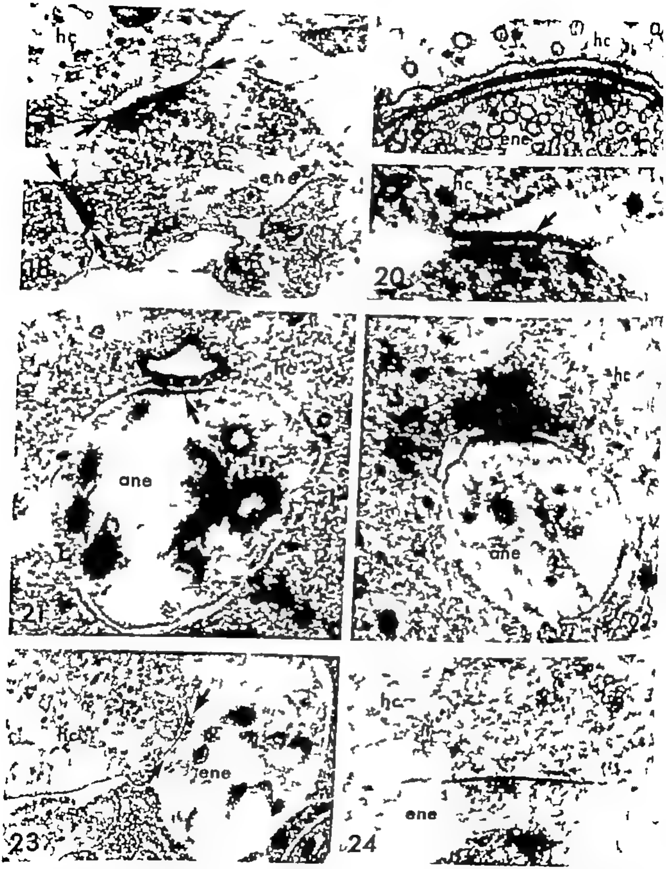

Fig 18 Survey picture of an efferent nerve ending in glutaraldehyde-fixed double-stained tissue Two synapses with two separate hair cells are seen on the left side of the efferent nerve cross-section (each circumscribed between two arrows) Only silhouettes of hair cell and nerve membranes are faintly outlined ×41 000

Fig 19 Efferent synapse in osmium tetroxide-fixed double stained tissue Note the electron-dense periodicity

Szamier & Wachtel 1970) and in the ampulla of Lorenzini (Barets & Szabo 1962, Waltman, 1966; Derbin 1970 1974 Jorgensen et al 1972). Similar structures are present also in photoreceptor cells and bipolar terminals in the retina (Ladman 1958 Sjöstrand 1958 Gray & Pease 1971) and in the pineal gland where they are not confined to the synaptic zones (Vollrath & Huss 1973)

The functional role of the synaptic bodies remains unknown, but their frequent presence at afferent synapses in sense organs suggests that they perform a similar function in all cases. One interesting observation is the variation in size and numbers of synaptic ribbons depending upon the level of excitation in the pineal body (Wagner 1973 Vollrath & Huss 1973 Vollrath & Howe 1976). Also in the ear it has been seen to grow in size in response to acoustical stimulation in the frog basilar papilla (Frishkopf 1973). In lateral line organs of salamander tadpoles where the synaptic bodies can be seen in vivo in its *transparent* tail (Flock et al 1973) they are seen to move to the base of the cell with a slow time course and also to change in numbers (Flock 1974). All this indicates some role in the plasticity of sensory synapses. Afferent synapses are required to transmit graded well-controlled signals from the sensory cell to the nerve fibre rather than all-or-nothing events as in synapses of the CNS and in the muscle end plate. It is possible that the synaptic body is engaged in controlling the guidance and access of synaptic vesicles to the presynaptic membrane. When in strong demand as during continuous excitation, the synaptic bodies may be capable of increasing the transmitting capacity of the afferent synapse by an increase in size or in numbers. Synaptic bodies develop in hair cells independent of innervation (Delaveuve 1974 Jørgensen & Flock 1976). It appears that during innervation and reinnervation the synaptic body and afferent ending establish contact by some form of trophic influence (Derbin 1970; Glenner & Wersäll 1974). Synaptic bodies floating freely in the cytoplasm in adult organs as described here and in the lateral line of amphibia (Wickham 1974) are surrounded by synaptic vesicles as if they were attached.

Enzymatic digestion of synaptic bars of the retina shows that the bars are constructed from proteins (Bunt 1971). In the inner ear it was found that the electron density of the synaptic body was affected by monoamine depleting drugs (Osborne & Thornhill 1972). However the afferent transmitter is not likely to be a catecholamine (Klinke & Evans 1977) or GABA (Klinke & Oertel 1977*a*. Sand et al 1975) as suggested by previous work (Flock & Lam 1974) or 5-HT (Klinke & Oertel 1977*b*). Amino acids are still *possible* candidates (Steinbach & Bennett 1971 Klinke & Oertel 1977*c*). A possible role for the synaptic body as a site for calcium-binding in relation to synaptic transmission has also been investigated in lateral line hair cells and rejected (Benshalom & Flock, 1977)

A new perspective on the possible mechanisms involved in synaptic function has been opened up by the recent demonstration of an *actin-like* protein associated with the post synaptic membrane in synapses from the CNS (Blomberg et al 1977). Non-muscle actin has been shown to participate in the motion of cellular components and also in the communication between membrane proteins facing the *extracellular* space such as receptors and *intracellular* subsurface structures (for references see Blomberg et al 1977). The continued search for the role of the synaptic body demands identification and characterization of its protein composition by sophisticated biochemical and immunological methods.

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material the embedding procedure rather than the glutaraldehyde is thus to blame The electron-dense inner coat of the postsynaptic membranes is separated from the outer layer of the nerve membrane at the afferent synapse The membrane at the efferent synapse is split into two leaflets

In non-osmicated preparations a denser sheet around the synaptic body s feet is revealed It has been assumed that tissue fixed with aldehyde without postfixation is less stable and resistant to dehydration so that distortions can occur (Schultz & Karlsson 1965) At the same time the differences noticed in the glutaraldehyde-fixed tissue may reveal true differences in the chemical composition of these membranes that were masked by the osmication procedure (Sabatini et al 1963 Oschman & Wall 1972) Such differentiation is seen here postsynaptically rather than presynaptically and does not apply to the particle-studded membrane seen in freeze fracture

E PTA which has been used for the selective staining of synapses in the brain (Bloom & Aghajanian 1972) appeared to react with a similar specificity at the afferent and efferent synapses on hair cells In both types of synapse postsynaptic membrane components are prominent even though in the efferent synapse they are part of the hair cell membrane This fact emphasizes that the presence of E PTA stained material is related to a particular function in that membrane rather than to its belonging to a particular cell type Given the present findings it would be of interest to test the E PTA staining properties of the afferent synapses on outer hair cells in the cochlea that are unique in having symmetric membrane complexes and in lacking synaptic ribbons (Dunn & Morest 1975)

Occasionally the presynaptic membranes are faintly stained by E PTA in both types of synapses while the synaptic vesicles are not seen At the same time the presynaptic projections of the efferent synapse are clearly stained This suggests that the electron-dense projections are neither synaptic vesicle condensations nor dumps of synaptic vesicles (Spoendlin 1973 Nakai & Hilding, 1969) Their presence at the presynaptic membrane (Wersäll et al 1965) and their shape and affinity for E PTA suggest that the projections are of the same type as those described in synapses of parts of the brain (for reviews, see Akert et al 1969 Pfenninger 1973). It has been suggested that these projections are derived from shells of the complex vesicles (Gray & Willis 1970)

Observations on synapses in the CNS using freeze fracture techniques have led to the alternative hypothesis that the presynaptic projections serve as vesicle attachment sites (Pfenninger & Rovainen 1974) At lateral line efferent synapses we have not been able to discern a highly organized presynaptic vesicular grid as described at CNS synapses (Akert et al 1969)

The synaptic body which appears quite homogeneous after osmium tetroxide fixation, is differently stained by E PTA Its feet and the outer layer of its main mass are electron-dense while its main core is electron-lucent This may mean that the core of the synaptic body has a different composition than the shell The whole of the synaptic body maintains its affinity to lead and uranyl acetate in section staining which shows that the core substance was not extracted during E PTA treatment PTA staining of synaptic bodies has also been described in the frog s labyrinth (Gleisner et al 1973) and the lateral line organ of Xenopus (Delaveuve & Szabo 1966)

Electron-dense bodies of variable sizes and shapes (bars ribbons sheets spheroids, etc) have been described at afferent synapses in sensory receptors of the acoustic and vestibular systems as well as in several types of sense organs related to the inner ear such as the lateral line organ of other fishes (Hama 1965 Hama & Saito 1977 Roberts & Ryan 1971) and amphibia (Jande 1966 Shelton 1971 Jorgensen & Flock 1973) or their related organs the electroreceptors of fishes (Mullinger 1964 1969 Szabo & Wersäll 1970

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ZUSAMMENFASSUNG

An den Haarzellen in dem Seitenlinienorgan des Fisches *Lota lota* wurde die Ultrastruktur der afferenten und efferenten Synapsen mit verschiedenen Fixierungs- und Farbungsmethoden untersucht Aus den Resultaten der Untersuchungen wurden neue Aufschlusse uber das auf der Membran aufgelagerte Material insbesondere über den präsynaptischen Körper und die postsynaptischen Verdickungen gewonnen Das Material war mit Glutaraldehyd fixiert worden jedoch nicht mit Osmiumtetraoxid behandelt worden Die Kontrastwirkung wurde statt dessen durch Schnittfärbung mit Uranylazetat und Bleizitrat oder durch Blockimprägnierung mit Phosphowolframsäure (phosphotungstic acid=PTA) erreicht Die PTA Färbung verstarkt das Hervortreten sowohl der afferenten wie der efferenten Synapsen an der postsynaptischen Membran Durch die Blockimprägnierung mit PTA werden auch die präsynaptischen dichten Projektionen an der afferenten Synapse und in differenzierender Weise der afferente synaptische Korper gefärbt Die Schnittfärbung laßt deutlich eine Substruktur in den Fußchen des afferenten synaptischen Körpers erkennen wahrend sie in mit Osmiumtetraoxid behandeltem Gewebe nur schwach angedeutet ist

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NEURONAL HABITUATION IN THE VESTIBULAR NUCLEI OF THE CAT

P Kileny, J H Ryu,[1] B F McCabe[2] and P J Abbas

From the Department of Speech Pathology and Audiology and of [2]Otolaryngology and Maxillofacial Surgery, University of Iowa, Iowa City, Iowa, USA

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Abstract This is a study of the effects of repeated angular acceleration on the activity of vestibular nuclei units in anesthetized and in conscious cats. The experimental animals were subjected to trains of repeated and consecutive constant acceleration–deceleration ramps (4–8°/sec²). In a few cases nystagmus was recorded along with extracellular unit activity.

The effect of stimulus repetition on vestibular neuronal activity consisted of one of the following:

1 No change in response.

2 Progressive response decline.

3 An initial maintenance of a constant response level throughout the first part of the stimulus paradigm, followed by a progressive decline.

4 An initial gradual enhancement of response followed by a progressive decline.

This classification is based on results of polynomial and non-linear broken line regression analyses. Categories 3 and 4 were found predominantly in units recorded from conscious cats. The majority of neurons recorded from anesthetized cats that exhibited response changes upon stimulus repetition manifested a progressive response decline. The response decline in anesthetized cat units was usually on a faster time scale than in conscious cats. Correlations between unit response modification patterns and simultaneously recorded nystagmus were mostly of moderate degree.

A progressive decrement of behavioral or neural response occurring with repeated presentations of an effective stimulus in the presence of an unaltered capacity to respond is defined as the habituation of the response. Under controlled conditions (allowing no interference with the transmission of the stimulus) the re-occurrence of the stimulus appears to be the sole extrinsic factor responsible for the diminution of the response. As with other plastic behavioral phenomena (i.e. classical conditioning) this process establishes temporarily a *new stimulus*–response relationship. Habituation is considered to be an elementary form of behavioral plasticity (Thompson & Spencer 1966), a fundamental adaptive mechanism (Pribram, 1967) or the simplest form of learning (Groves & Thompson 1973). Responses subject to habituation range from behavioral to single unit activity. All are bound together by a series of common characteristics which set habituation apart from other decremental phenomena (adaptation or end-organ fatigue) such as specificity to modality and to spatial and temporal configuration of the input (Thompson & Spencer 1966).

With repeated vestibular endorgan stimulation there occurs a modification of the vestibulo-ocular reflex, the most common effect being a progressive decline in the magnitude of the various nystagmus parameters (Fernandez & Schmidt 1962, Lidvall 1961, Forssman, Henriksson & Dolowitz, 1963, Henriksson, Kohut & Fernandez, 1961, Collins 1964, 1974). This phenomenon occurs with both angular acceleration and caloric stimuli and the final outcome is affected by extravestibular factors such as visual input and degree of arousal (Collins 1964, 1974).

In spite of growing evidence of habituation of neuronal responses in sensory and motor systems (Horn 1970, Groves & Thompson 1970, Segundo, Takenaka & Encabo 1967, Buchwald & Humphrey 1973, Peterson et al 1976), there have been few attempts to investigate this phenomenon in the vestibulo-ocular

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[1] Present address: Speech Pathology, Audiology Department, Glenrose Hospital, 10230-111 Avenue, Edmonton, Alberta T5G 0B7, Canada.

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Åke Flock M.D
Dept of Physiology II
Karolinska Institutet
S 10401 Stockholm
Sweden

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NEURONAL HABITUATION IN THE VESTIBULAR NUCLEI OF THE CAT

P. Kileny, J. H. Ryu, B. F. McCabe[a] and P. J. Abbas

From the [1]Departments of Speech Pathology and Audiology and of [2]Otolaryngology and Maxillofacial Surgery, University of Iowa, Iowa City, Iowa, USA

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Abstract. This is a study of the effects of repeated angular acceleration on the activity of vestibular nuclei units in anesthetized and in conscious cats. The experimental animals were subjected to trains of repeated and consecutive constant acceleration–deceleration ramps (4–8°/sec²). In a few cases nystagmus was recorded along with extracellular unit activity.

The effect of stimulus repetition on vestibular neuronal activity consisted of one of the following:

1. No change in response.
2. Progressive response decline.
3. An initial maintenance of a constant response level throughout the first part of the stimulus paradigm, followed by a progressive decline.
4. An initial gradual enhancement of response followed by a progressive decline.

This classification is based on results of polynomial and non-linear broken-line regression analyses. Categories 3 and 4 were found predominantly in units recorded from conscious cats. The majority of neurons recorded from anesthetized cats that exhibited response changes upon stimulus repetition manifested a progressive response decline. The response decline in anesthetized cat units was usually on a faster time scale than in conscious cats. Correlations between unit response modification patterns and simultaneously recorded nystagmus were mostly of moderate degree.

A progressive decrement of behavioral or neural response occurring with repeated presentations of an effective stimulus in the presence of an unaltered capacity to respond is defined as the habituation of the response. Under controlled conditions (allowing no interference with the transmission of the stimulus) the re-occurrence of the stimulus appears to be the sole extrinsic factor responsible for the diminution of the response. As with other plastic behavioral phenomena (i.e. classical conditioning) this process establishes temporarily a new stimulus–response relationship. Habituation is considered to be an elementary form of behavioral plasticity (Thompson & Spencer, 1966), a fundamental adaptive mechanism (Pribram, 1967) or the simplest form of learning ((Groves & Thompson, 1973). Responses subject to habituation range from behavioral to single unit activity. All are bound together by a series of common characteristics which set habituation apart from other decremental phenomena (adaptation or end-organ fatigue) such as specificity to modality and to spatial and temporal configuration of the input (Thompson & Spencer, 1966).

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In spite of growing evidence of habituation of neuronal responses in sensory and motor systems (Horn, 1970; Groves & Thompson, 1970; Segundo, Takenaka & Encabo, 1967; Buchwald & Humphrey, 1973; Peterson et al., 1976), there have been few attempts to investigate this phenomenon in the vestibulo-ocular

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pathway (Crampton 1965 Goetemakers 1970)

The purpose of this study was to investigate the effects of repeated and consecutive accelerations and decelerations on vestibular nuclei neurons in anesthetized and unanesthetized intact cats

METHODS

Experimental animals and their preparation

A total of 38 cats each weighing between 2 0 and 4 0 kg were used in the present study Preoperative and operative procedures were identical for all experimental animals and are described elsewhere (Ryu & McCabe 1973) In 19 cats unit recording was carried out under general sodium-pentobarbital anesthesia. In a second group of 19 unit recording was carried out unanesthetized under Pancuronium Bromide (Pavulon® Organon) induced paralysis or as *encéphale isolé* preparations Both preparations necessitated artificial ventilation Recording was performed with tungsten or glass pipette electrodes driven through an opening in the dura mater through the intact cerebellum to the approximate region of the vestibular nuclei following stereotaxic coordinates (Berman 1968) After completion of the craniotomy in the unanesthetized group an L shaped brass plate was mounted on the frontal bone the scalp sutured back. The cats were allowed to recover from the operation for 2 to 7 days

On the day of the experiment a tracheostomy was performed under ether anesthesia and the cats were mounted in the stereotaxic frame A bar mounted on the stereotaxic frame was fastened to the L shaped plate mounted over the frontal sinus after the cat was already in the stereotaxic position This allowed the removal of the eye bars tooth and the ear bars without altering the cat s position The wounds were periodically infiltrated with 1% Lidocaine Hydrochloride

The stimuli consisted of consecutive constant horizontal clockwise angular accelerations followed by equal clockwise decelerations (4–8°/sec²) Initial velocity was always zero The peak velocity at the end of an acceleration was 90 or 100 degrees per second

Extracellular unit activity and induced nystagmus were evaluated for progressive response alterations occurring with stimulus repetition In order to determine the stability of neurons spontaneous resting discharge was recorded for 1 024 sec This was followed by the experimental paradigm consisting of up to 70 repeated and consecutive accelerations and decelerations If a decline in neuronal response was detected dishabituation was usually attempted by dropping out one or two consecutive stimuli from the sequence or by replacing the standard acceleration–deceleration stimulus by a new one such as calorics The response to a given stimulus was evaluated by integrating total discharge above average resting discharge rate during the stimulation period of interest The perimeter of the area circumscribed by the discharge rate histogram time locked to stimulus period was traced with the probe of a sonic digitizer and an area corresponding to response magnitude was obtained Nystagmus was retrieved as the electrooculographic recording of eye displacement and total slow phase displacement was measured manually from these records for every acceleration and deceleration

RESULTS

Units in anesthetized cats

Thirty five vestibular nuclei units recorded stereotaxically from 19 anesthetized cats were examined for response modification An examination of the series of discharge rate histograms elicited by the repeated and consecutive acceleration–deceleration stimuli revealed seventeen units with no detectable response modification The rest were divided into two apparently distinct patterns of response modification either the response declined pro-

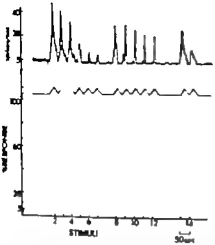

Fig. 1. Habituation, dishabituation and rehabituation of an unselected cat unit: successive discharge rate histograms (*upper trace*), angular velocity waveforms (*second trace*) and percentage response change (*lower graph*).

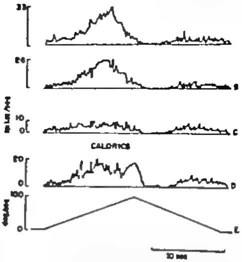

Fig. 2. The effect of ice water calorics on depressed neuronal response following stimulus repetition. (A) Averaged discharge rate histogram elicited by stimuli 1–8. (B) Averaged discharge rate histogram elicited by stimuli 9–16. (C) Averaged discharge rate histogram elicited by stimuli 32–40. (D) Averaged discharge rate histogram elicited by stimuli 41–48 following ice-water calorics.

sively with stimulus repetition as shown in Fig. 1 (14 units) or there was an apparent initial increase in response magnitude followed by a progressive decline. In Fig. 1 and some of the following figures the upper trace is the frequency discharge histogram elicited by repeated and consecutive accelerations and decelerations. Stimulus velocity waveforms are illustrated in the middle trace. The bottom graph plots the percentage response (with respect to the second response) vs stimulus number.

Dishabituation of the habituated neuronal response was attempted by a break in stimulation equivalent in duration to 1–3 stimuli in fourteen neurons. In seven of these dishabituation was attempted a second time with a second break. In four neurons dishabituation was attempted by interposing a new stimulus calorics. The effect of interrupting the stimulus train is illustrated in Fig. 1. A short break equivalent in duration to one standard acceleration–deceleration stimulus was effective twice in partially restoring the response. After six repetitions of the acceleration–deceleration stimulus the response declined to about 10% of control. Following this one stimulus was omitted from the sequence. The second stimulus following the short break elicited a response that was 50% of the control. With further stimulation the response dropped down to 25%. Another break equivalent in duration to two stimuli again restored the response partially.

The effect of ice water calorics on a habituated neuronal response is illustrated in Fig. 2. Each discharge rate histogram shown in this figure represents the sum of eight responses of a unit to consecutive 8°/sec² acceleration–deceleration stimuli. The angular velocity waveform of one acceleration–deceleration is shown at the bottom of Fig. 2 (E). A, B and C are the first, second and fifth groups of eight responses. There is an obvious decline from A to C in both the area circumscribed by the discharge rate histogram and its amplitude. The discharge rate histogram designated (D) represents the sum of eight responses obtained following the delivery of 10 cc ice water in the left ear of the anesthetized cat, the recovery is evident.

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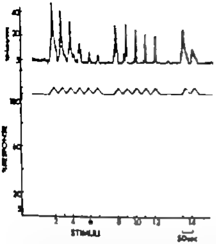

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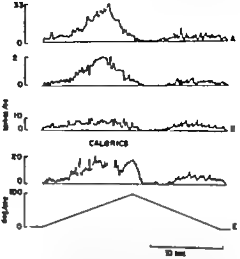

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Table I *Summary Trend analysis*

	Number of cases			
	Nonsig.	Linear	Quadr	Quadr & Lin.
Anesth. unit	17	13	1	4
Consc unit	16	7	3	17

$\chi^2=9.42$ (significant at 0.01 level).

stimuli. During the last eleven stimuli unit activity declined substantially

In eight units nystagmus was recorded simultaneously with unit activity for time segments of various lengths. Fig. 6 illustrates a typical example of simultaneous changes of neuronal and nystagmus response expressed as percentage response. The two curves appear similar although the percentage change in activity was larger for the unit (upper portion of the graph) than for nystagmus. Both nystagmus and unit response increased initially and then decreased back to about control level.

In order to determine the statistical significance of the observed response alterations and to provide an objective means for grouping the various response modification patterns and describe them mathematically the data were submitted to a number of statistical analysis procedures.

Polynomial regression or trend analysis determined the general trend. Unit response modification patterns were divided by trend analysis into linear and linear & quadratic (second-order polynomial) trends. The majority of anesthetized units that did manifest response modification with stimulus repetition were classified as 'linear'. About two-thirds of the units from conscious cats were linear and quadratic (second-order polynomial) and one-third linear. A χ-square significant at the 0.01 level confirmed the significant difference between the two groups of units. Results of polynomial regression are summarized in Table I. For the 'linear' units the slope of the regression line was also calculated.

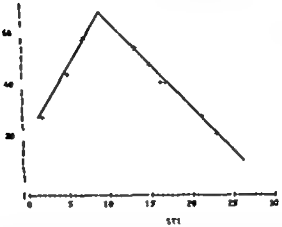

Fig. 7. Non-linear broken-line regression analysis performed on the response alteration pattern with respect to successive stimuli of conscious cat unit. X-axis: successive stimuli; Y-axis: digitized area under discharge rate histogram in mm². A (positive slope)=12.65%. B (negative slope)=−5.02%.

In order to determine whether the initial increase and the subsequent decrease observed in the units defined by a second-degree polynomial were significant, data from those units

Table II *Summary Regression analyses*

	Lin. regr Neg. slope		Non-lin. regr 1				Non-lin. regr.2		
			Pos. slope		Neg. slope		Pos. slope	Neg. slope	
	Mean	S.D.	Mean	S.D.	Mean	S.D.		Mean	S.D.
Anesth. unit	−8.84 N 14	6.75	11.02 N=1		−16.60		Non-sig.	−8.27 N=3	1.01
Consc unit	−2.72 N=8	1.95	8.52 N=8	10.39	−6.38	2.53	Non-sig.	−5.90 N=11	3.76

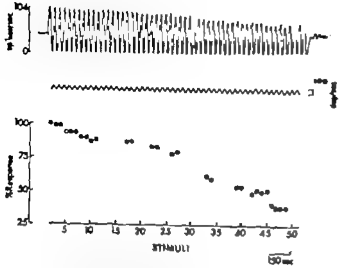

Fig 3 Progressive response decline manifested by a conscious cat unit with stimulus repetition *Upper trace* Successive discharge rate histograms *Second trace* Angular velocity waveform. *Lower graph* Percentage response change

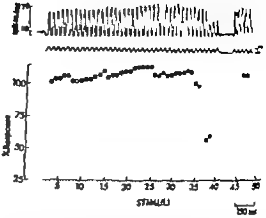

Fig 5 Response decline with repeated stimulation a recovery following a short break equivalent in duration three acceleration–deceleration stimuli in a conscious c unit: Successive discharge rate histograms, stimul waveform and percentage change in response with su cessive stimuli.

Units in conscious cats

Forty two units recorded from 19 unanesthetized cats were examined for response modification Nystagmus was recorded simultaneously with eight units from five *encéphale isolé* preparations In twenty units the paradigm was repeated twice

Inspection of the data suggested three apparently distinct patterns of response modification The unit illustrated in Fig 3 shows typical progressive and orderly respons decline with stimulus repetition This patter was on a slower time scale than its equivaler found in anesthetized cats Fig 4 illustrate responses from a typical unit showing an initi gradual increase in its evoked activity followe by a progressive decline The third pattern i illustrated in Fig 5 This unit did not alter it activity substantially with the first thirt

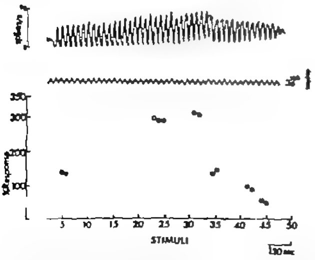

Fig 4 An initial response enhancement followed by a progressive decline manifested by a conscious cat unit with stimulus repetition *Upper trace* Successive discharge rate histograms. *Second trace* Angular velocity waveform *Lower graph* Percentage change in response

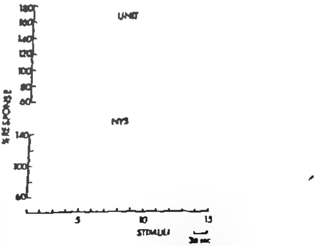

Fig 6 Simultaneous changes in nystagmus and unit response with repeated stimulation Both are expressed as percentage response -0.69; $p=0.02$.

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Anesth. unit[a]	17	13	1	4
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χ^2=9 [illegible] (significant at 0 01 level).

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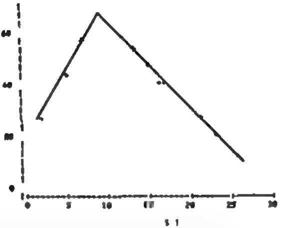

Fig 7 Non-linear broken-line regression analysis performed on the response alteration pattern with respect to successive stimuli of conscious cat unit. X-axis, successive stimuli; Y-axis digitized area under discharge rate histogram in mm² A (positive slope) 12.65% B (negative slope)= −5.02%

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	Lin. regr Neg slope		Non-lin. regr 1				Non-lin. regr.2		
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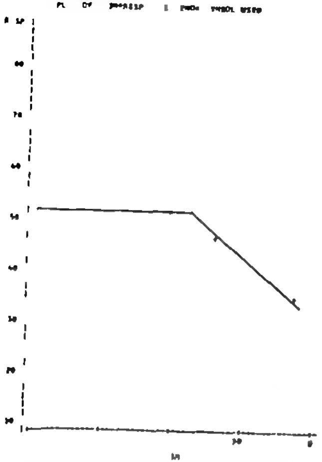

Fig 8 Non-linear broken-line regression analysis performed on the response alteration pattern with respect to successive stimuli of a conscious cat unit X-axis: successive stimuli Y-axis, digitized area under discharge rate histogram, in mm²

were also subjected to a non linear broken line regression analysis which fits the data with two straight lines having different slopes and intercepts (Gallant 1975 Barr et al 1976) The slope of each line and the break point were estimated A 95% confidence interval was specified for each slope estimation This served as a test of significance The results of this procedure are summarized in Table II

In the next two figures which illustrate the results of the broken-line regression analysis the abscissa is scaled by successive stimuli of equal duration The ordinate is scaled by digitized area units proportional to the areas circumscribed by successive discharge rate histograms

Fig 7 illustrates the result of the broken line non linear regression analysis performed on a unit whose response modification was as-

Table III *Summary of nystagmus unit correlation*

Cat	Unit no	r	Sign. r
49	1	0 69*	0 05
	2	0.33	Non-sig.
51	1	0.543	0 05
	16	−0 10	0 05
46	2	0.24	0 05
	5	0 44	0.05
29	1	0.56	0 05
32	6	0.55	0 05

* Significant correlations.

sessed as being a second-degree polynomial. It was judged that this unit manifested a significant increase in response followed by a significant decline Fig 8 illustrates a unit with initially unmodified response followed by a significant decline

The relationship between nystagmus and unit response modification for the cases where both were simultaneously recorded was evaluated by calculating Pearson correlation coefficients for the pairs of simultaneously recorded response modalities In three of the eight cases where this was performed the correlation was found to be not significant. In the remaining five cases the correlation coefficients ranged between 0 44 and 0 69 and were found to be significant In the case illustrated in Fig 6 the correlation coefficient was 0 690 and was found to be significant Table III summarizes the correlations between nystagmus and unit activity

DISCUSSION

The vestibular nuclei act as a relay station between the vestibular endorgans and the CNS. It appeared appropriate therefore to investigate neuronal habituation at their level Sev eral studies have provided evidence that changes are likely to occur in the vestibular nuclei during the habituation of the vestibulo-ocular reflex The generalization (transfer) of

habituation from the trained to the untrained ear when eliciting nystagmus in the practiced direction (Henriksson et al., 1961; Fluur & Mandel, 1964) implicates the nuclear complex. Visual input has a marked effect upon habituation (Collins, 1967) and there is evidence for the modulating influence of visual input upon vestibular nuclei units (Henn et al., 1974). In addition, McCabe & Gillingham (1964) showed dishabituation following lesions to the lateral vestibular nucleus.

Over 50% of neurons recorded from the anesthetized cats manifested significant changes with stimulus repetition. Dishabituation was also demonstrated in a number of cases. While most neurons recorded from the anesthetized cats showed relatively rapid and unimodal changes in response magnitude with repeated stimulation, the changes seen in the conscious cat units were on a slower time scale. In addition, in a larger number of units were those changes bimodal. In approximately two-thirds of units recorded from the conscious cats, response decline was preceded by an enhancement, or by a period of constant response.

An initial response increment followed by a gradual decline occurring with repeated stimulation as observed in certain conscious cat vestibular neurons has been reported to occur also in other systems with stimulus repetition. Thus, during the habituation of the hindlimb flexor reflex in acute spinal cats, one group of interneurons exhibited an initial response increase followed by a gradual decline (Groves & Thompson, 1973), this being also the pattern of the reflex modification. The investigators attributed this patern to a competitive interaction between incremental and decremental processes generated at spinal interneuronal level. Some reticular neurons exhibited the same behaviour with repeated stimulation of tectal, vestibular cortical and cutaneous areas (Peterson et al., 1976). The increment was attributed to a longlasting facilitation through intrinsic reticuloreticular pathways. In the vestibular system some primary afferents in the frog manifested an exponential response decline with stimulus repetition, others maintained a constant response initially, which subsequently declined (Goetemarkers, 1970).

A simple model can be hypothesized. Initially the repeated acceleration–deceleration stimuli elicit a general arousal reaction, which contributes towards tonic facilitation of certain vestibular nuclei neurons through the reticular system (Duensing & Schaefer, 1957; Dumont, 1960; Bizzi et al., 1964). Hence the initial increase in response, or the maintenance of a constant response in conscious cat units. Under barbiturate anesthesia, both ascending and descending impulses through the reticular system are blocked (Magoun, 1958; Arduini & Arduini, 1954). Due to the relative inactivity of the reticular system in anesthetized preparation, an initial significant response increment was not prevalent in our anesthetized cats.

After a number of stimulus repetitions, the non-specific arousal reaction habituates (Sharpless & Jasper, 1956) and the vestibular nuclei lose the excitatory input associated with the arousal reaction process. At this point, the inhibitory cerebellar input (Ito et al., 1970; Robinson, 1976; *Singleton, 1967*) which may have been overshadowed by the excitatory reticular input, may become effective, and a progressive response decline may take over.

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ZUSAMMENFASSUNG

Die Effekte der wiederholenden winkligen Beschleunigung auf die Aktivität der Vestibulariskerne wurden in anästhesierten und bewußten Katzen studiert. Die Versuchstiere wurden Folgen von wiederholenden und nachfolgenden konstanten Beschleunigungs-Verlangsamungsrampen (4–8°/sec²) ausgesetzt. Der Nystagmus wurde in einigen Fällen zusammen mit extracellulärer Neuronenaktivität registriert.

Der Effekt der Wiederholung der Reize auf die vestibulare Neuronenaktivität war einer der folgenden

1 Keine Änderung in der Reizbeantwortung.

2. Eine progressive Verminderung der Reizbeantwortung.

3 Eine anfängliche Erhaltung eines konstanten Reizbeantwortungsspiegels während des ersten Teils der Reizparadigma mit einer progressiven Verminderung folgend

4 Eine anfängliche graduelle Steigerung der Reizbeantwortungen mit einer progressiven Verminderung folgend.

Diese Klassifikation beruht auf Ergebnissen von polynomen Regressionanalysen Die Kategorien 3 und 4 wurden vorwiegend in Neuronen bewußter Katzen registriert Die Mehrzahl der Neuronen die von anästhesierten Katzen registriert wurden und welche Änderungen der Reizbeantwortungen mit der Wiederholung des Reizes zeigten erwiesen eine progressive Verminderung der Reizbeantwortung. Die Verminderung der Reizbeantwortung in Neuronen von anästhesierten Katzen erschien meistens auf einem schnelleren Zeitmaßstab als in jenen von bewußten Katzen Die Korrelationen zwischen dem Modifikationsmuster der neuronen Reizbeantwortung und des gleichzeitigen registrierten Nystagmus waren meistens von gemäßigtem Grad.

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Dr P Kileny
Speech P thology–Audiology Dept
Glenrose Hospital
10230-111 A enue
Edmonton Alberta
T5G 0B7 Canada

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Dr P Kileny
Speech Pathology–Audiology Dept
Glenrose Hospital
10230-111 Avenue
Edmonton Alberta
T5G 0B7 Canada

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ANALYSIS OF HUMAN VESTIBULO-OCULAR REFLEX DURING ACTIVE HEAD MOVEMENTS

R D Tomlinson [1] G E Saunders [2] and D W F Schwarz [1]

From the [1]Department of Otolaryngology University of Toronto Toronto Ontario and the [2]Division of Medical Computing University of Toronto Toronto Ontario Canada

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Abstract The human vestibulo-ocular reflex (VOR) was investigated during active head movements utilizing spectral analysis techniques in order to extract phase and gain characteristics for the most natural stimulus conditions. Three different experimental conditions were examined. 1) head rotation in darkness to obtain data permitting a comparison with that mode of VOR analysis which has been most frequently employed in the past 2) head rotation while fixating a stationary target light in order to quantify natural compensatory eye movements and 3) head rotation while fixating a target light which moved with the head as a fast method for the quantification of visuo-vestibular interaction High frequency head rotation in darkness yielded gains not significantly different from unity—unlike previously reported results for passive rotation (Benson 1970 Keller 1978) Possible mechanisms which might explain these results are discussed

The phase and gain characteristics of the VOR (vestibulo-ocular reflex) have been extensively investigated in both humans and non human primates These investigations have generally involved passive rotation of the subject. This technique suffers from two basic problems 1) motor torque limitations have generally forced testing to be carried out at frequencies well below those involved in most normal head movements and 2) there is insufficient evidence as to how much non vestibular influence is involved in the compensatory eye movements seen

It is known for example that the visuomotor drive which contributes to compensatory eye movements during slow head movements can also operate in the dark (Barr et al 1976) Also the possibility of a contribution by the motor program for head movements to the fine regulation of compensatory eye movements is generally ignored although motor programs have been shown to operate for this purpose in pathological cases (Dichgans et al 1974)

In order to investigate these questions further a computer technique has been developed which allows measurement of VOR phase and gain characteristics during active non sinusoidal head movements The purpose of this paper is to describe this technique and present results detailing the interaction of vestibular and other inputs in the generation of compensatory eye movements

METHODS

Results were obtained from 6 healthy adults (3 male 3 female) between 20 and 35 years of age All had excellent uncorrected vision and were free of neurological and vestibular defects Voluntary lateral head movements were tested in three experimental conditions 1) in darkness (wearing light tight goggles in a dark room) 2) while viewing a stationary visual target (a red light (LED) of 2 mm diameter) located 1 meter in front of the head rotation axis and 3) while viewing an identical visual target attached to the head frame 25 cm in front of the eyes and moving with the head

All subjects were dark adapted for at least 30 min before the first trial During each experimental trial the subject performed 40 sec

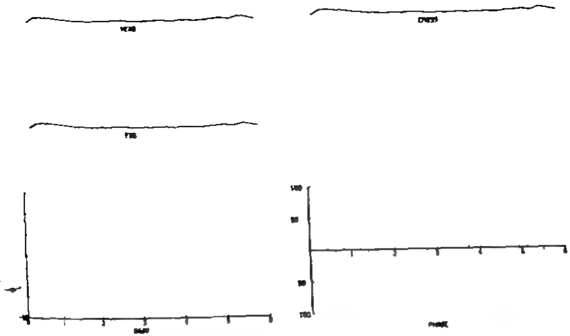

Fig. 1. Bode plots and power spectra of VOR during active head shaking with stationary visual target. Curves labeled *HEAD*, *EOG* and *CROSS* represent the head, eye, and cross power spectra respectively. Graphs labelled gain and phase show VOR performance plotted as a function of frequency for one 40 sec trial. Numbers on the ordinate denote frequency. Gain is plotted as

$$10 \log \frac{\text{peak-to-peak eye displacement}}{\text{peak-to-peak head displacement}}$$

of self-paced continuous headshaking at various amplitudes and frequencies. For testing, subjects were seated on an adjustable height chair and the head was firmly fixed in a light-weight aluminum frame fitted with a dental bite board and an adjustable headband. This frame could be rotated about a vertical axis passing approximately through the center of the head. A precision continuous turn linear potentiometer with a linearity of 0.1% was coupled to this axis in order to measure head position. The EOG was recorded with Beckman skin electrodes attached to the outer canthus of each eye and an indifferent electrode above the bridge of the nose. The EOG signal was amplified by a low drift d.c. differential amplifier and filtered at 100 Hz. Both EOG and head position signals were digitized at 200 Hz and stored on tape for further processing.

After each experiment the data were displayed on a graphics terminal, the saccades marked and removed to generate a cumulative eye position signal (Meiry 1966). The cumulative eye position and head position data were then filtered with a zero phase shift, 4-pole digital low-pass filter whose response was down 1 dB at 6 Hz. The eye position data were then fitted with a third-order polynomial which was subtracted out in order to minimize low frequency errors caused by voltage drift at the electrode. Finally, power spectral analysis techniques were used to calculate the phase and gain of the reflex at all frequencies having a sufficient power content. The digital techniques are described in greater detail in the Appendix.

This technique has proved useful in the experimental laboratory and, more importantly, in the clinic, to quantify VOR performance throughout the physiological head rotation range within a very short time. Advantages attributable to avoidance of sinusoidal stimuli are evident in this report.

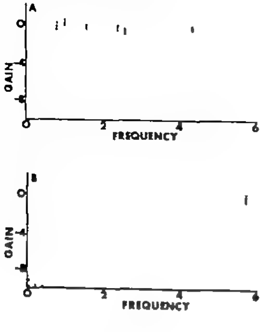

Fig 2 Superimposed gain curves of 6 normal subjects (A) Head rotation in darkness. (B) Head rotation while attempting to suppress VOR by fixating a target moving with the head (C) Head rotation while attempting to fixate a stationary target.

RESULTS

(A) *Normal compensatory eye movements*

During head rotation in a steady visual environment compensatory eye movements are produced by a cooperation of various systems including the VOR and visual tracking. When subjects were instructed to shake their heads while observing a stationary target the results were as expected a unity gain (0 9 to 1 1) was observed at all but the highest frequencies of between 5 and 6 Hz where a small gain decrease was seen (Fig 1) Subjects reported that the target appeared as a short horizontal streak at these high frequencies thus providing a subjective perceptual correlate for the gain decrease Phase was constantly zero within EOG resolution

(B) *VOR suppression*

If a visual target moving with the head is fixated the VOR can be suppressed within the dynamic range of the smooth pursuit system, but not during high velocities of head rotation. This was evident in the records which were essentially identical for all 6 subjects (Fig. 2B) As can be seen from the gain curves when the subject was asked to track a target moving with the head VOR suppression was nearly perfect (gain near zero) up to a frequency of 0 5 Hz The gain then rose smoothly in the 0.5 to 2 0 Hz range until it reached a value of 1 0 for frequencies above 2.0 to 2.5 Hz. Phase angles were always small (less than ±10°) throughout the frequency range. There was a small phase lead (generally 5 to 8°) observed at frequencies below 3 Hz which changed to a small phase lag of about the same magnitude at frequencies above this value (Fig 3) Thus three ranges for compensatory eye movements can be identified a low frequency range within which the smooth pursuit system is dominant a high frequency range in which the non visual reflex drive (mainly the VOR) can completely override fixation (mainly attributable to the smooth pursuit system) and an intermediate range in which the interaction between vestibular and visual inputs is essential The recorded data are of course matched by subjective perception the red dot seen at low frequencies was reported to appear as an increasingly longer line at increasingly higher velocities until the length of the line seen corresponded to the amount of head deflection during very rapid oscillations

(C) *Compensatory eye movements in darkness*

Passive rotation in darkness has been widely used to characterize vestibular input In the low frequency range (Fig 2 A) our data compare well with previous reports indicating a gradual increase in gain with frequencies up to approx 1 Hz where a value of unity is reached There was no great variation of gains between

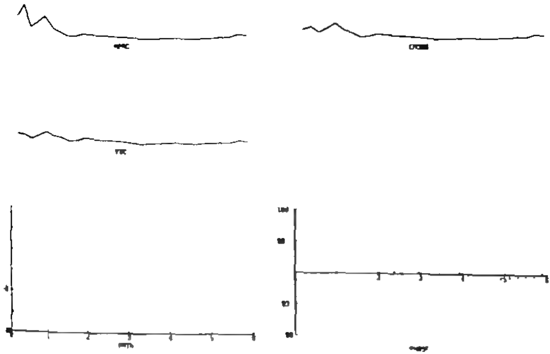

Fig. 3. Bode plots and power spectra during attempted VOR suppression. Phase estimates below 2 Hz are not significant, due to increased variance during active suppression.

subjects although no specific gaze instructions were offered. It is clear, however, that these low frequency gains can be altered up or downwards by appropriate gaze effort (Barr et al. 1976). This is not true for high frequencies where our data yielded a most unexpected observation.

It has previously been reported that humans (Benson 1970) and monkeys (Keller 1978) exhibit an increase in VOR gain above unity (up to 1.4 in some cases) during high frequency passive rotation. Keller reported that in *Macaca mulatta* the maximum gain was reached at 4 Hz and then began to fall while Benson's human subjects exhibited gains which were still increasing at 5 Hz, the highest frequency tested. In none of our subjects were any such gain increases seen. The gain reached its maximum value of unity at about 1 Hz and then remained stable until the high frequency limit was reached between 5 and 6 Hz, where the gain began to decrease. In addition to our failure to detect any gain increase at high frequencies, the reflex exhibited a small phase lag of about 6 to 8° above 3 Hz—much as was seen during VOR suppression.

DISCUSSION

The results of our experiments on VOR phase and gain during active head movements contain some interesting and unexpected findings. There can be little doubt, judging by the results of Benson (1970) and Keller (1978), that VOR gain increases above unity during high frequency passive rotation. Furthermore Keller reported that the low frequency phase lead was present up to the high frequency limits of the reflex. Our data, however, indicate that during active head rotation no gain increase is present and the low frequency phase lead is converted to a phase lag at about 3 Hz, well below the high frequency limit of 5 to 6 Hz.

There are two possible explanations for the differences between active and passive rota-

tions Neck proprioceptors are known to feed information through the vestibular nuclei to occulomotor neurons (Hikosaka & Maeda 1973) If the gain increase were caused by the high frequency lead element in the peripheral transduction process (Fernandez & Goldberg 1971) which is not quite matched by orbital lag elements (as suggested by Keller 1978) the result would be an overestimate of head velocity by the vestibular system in the absence of other information Neck proprioceptive information might conceivably function to increase the accuracy of the vestibular system s best estimate of head velocity and thus decrease the gain to the appropriate value Alternatively the motor program generating the head movement might be fed to the vestibular system according to the efference copy concept (von Holst 1954) and thus correct the head velocity estimate In either event our data demonstrate that the vestibular system s best estimate of head velocity and thus the gain of the VOR is markedly improved during active head rotations as compared with the performance during passive movements

The demonstration that compensatory eye movements are not only generated in response to the vestibular stimulation during head rotations is of course no novelty Dichgans et al (1974) have demonstrated the contribution of neck afferent input as well as the role of the head movement program particularly after labyrinthectomy These factors were differentiated by braking the head movement to a halt immediately after its initiation a procedure which cannot be used in human experimentation when head fixation is achieved via a bite board Since it seems impossible to rule out a contribution from neck receptors during active muscle contraction prevented from resulting in a head movement we made no attempt to separate these two factors

The demonstration that the visual tracking systems dominate the VOR at low frequencies confirms earlier reports (Barnes et al 1978) It is nevertheless in this low frequency range that analysis and indeed quantification of the VOR for diagnostic purposes is usually performed It is therefore of clinical importance to realize that low frequency gains of compensatory eye movements can only characterize the vestibular systems to the extent of the vestibular contribution The amount of extra-vestibular influence is rarely if ever known it is certainly not excluded by rotation in darkness (Barr et al 1976) If higher frequencies are used above 2 Hz these extra-vestibular influences may be minimized so that a much more precise quantification of vestibular function may be obtained

ACKNOWLEDGEMENT

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Appendix

The digital treatment of the data may be broken down into three sections 1) trend removal 2) digital filtering and 3) calculation of power spectra

Trend removal

If we represent the digitized data as X_i $i = A$ $A+1$ B for some arbitrary A and B with the constraint that the number of data points equals $N = B - A + 1$ then the data with a polynomial trend removed can be represented as $Y_i = X - (\sum_{k=0}^{K} b_k i^k)$ $i = A$ B where the coefficients b_k are to be determined Letting $Q(b) = \sum_i^B Y^2$ then the coefficients may be determined by setting the partial derivatives of Q with respect to each b to zero and solving the resulting equations i e

$$\frac{\partial Q}{\partial b_k} = -2\sum_{i=A}^{B} Y_i i^k \quad k = 0\ 1\ 2\ \ldots\ K$$

This set of simultaneous equations may be represented in matrix form as

$$\begin{bmatrix} B-A+1 & \Sigma i & \ldots & \Sigma i^K \\ \Sigma i & \Sigma i^2 & \ldots & \Sigma i^{K+1} \\ \vdots & & & \vdots \\ \Sigma i^K & \Sigma i^{K+1} & \ldots & \Sigma i^{2K} \end{bmatrix} \begin{bmatrix} b_0 \\ b_1 \\ \vdots \\ b_K \end{bmatrix} = \begin{bmatrix} \Sigma X \\ \Sigma X i \\ \vdots \\ \Sigma X i^K \end{bmatrix}$$

where

ΣI_k is an abbreviation for $\Sigma_{-N}I^k$

and

$\Sigma X I_k$ is an abbreviation for $\Sigma \quad X_i I_k$

The solution of this matrix equation will thus result in the appropriate b_k. In practice a polynomial of order 3 ($K=3$) was chosen as being the best compromise between optimal artifact removal and maximal speed of execution.

Digital filtering

Since the data collection rate must be far higher than required to represent the low frequency information that is of interest (because of the need to represent saccades well) time can be saved on the Fourier transform by applying a low-pass filter and then decimating the data. A digital Butterworth filter was obtained by bilinear transformation because of the sharp roll-off in the stop band and because it is possible to apply the transformation separately to the factored transfer function resulting in a zero-phase shift design

The zero-phase shift property can be seen by looking at the spectral representation of the time-reversed signal For a given signal $x(t)$ its Fourier transform is

$$X(\omega)=\int_{-\infty}^{+\infty} x(t)\, e_{-j\omega t}\, dt$$

and

$$\int_{-\infty}^{+\infty} x(-t)\, e_{-j\omega t}\, dt = {}_{-\infty}x(t)\, e_{j\omega t}\, dt = X\ (\omega)$$

is the transformation of the time-reversed signal Letting $H(\omega)$ be the frequency response of the filter and $X(\omega)$ the Fourier transform of the input signal, then the Fourier transform of the filter will be $Y(\omega)=H(\omega)X(\omega)$ and of the reversed signal $Y^*(\omega)=H\ (\omega)X\ \ (\omega)$ If we apply the filter to this signal we obtain a new signal with a Fourier transform $H(\omega)Y^*(\omega)=|H(\omega)|^2X\ (\omega)$ If we then take the time reverse of this we obtain $Z\ (\omega)=|H\ (\omega)|^2Y(\omega)$ Note that this signal although it has been low pass filtered by a magnitude $|H(\omega)|^2$ contains exactly the same phase information as the input Thus the filter has zero-phase shift characteristics and will not result in phase distortions of the data during filtering

Power spectral analysis

As might be expected data on human head shaking contain strong spectral peaks, which makes the reduction of leakage important. Nonetheless use of a severe data taper such as the Hanning window could broaden the main lobe sufficiently to introduce bias due to the averaging of a single spectral peak. For this reason a 10% split-cosine bell taper was deemed a good compromise

The data are decimated to produce as close to 512 data points as possible and are padded out with zeros The Fourier transform is then applied to the input and output signals simultaneously and the transforms are stored in two arrays of 256 points each which represent the spectra up to the folding (or Nyquist) frequency The spectra are then calculated according to the formulae

$$G\ (f\ )=\frac{2H}{N}|X_k|^2$$

$$G_{xy}(f_k)=\frac{2H}{N}(X\ \ Y\ )$$

where $N=512$

and H=interval between decimated dat points

Gain and phase values are then calculated

$$\text{Gain}=g=\left\{\left[\frac{\text{real}\ G_{xy}}{G}\right]^2+\left[\frac{\text{imaginary}\ G_{xy}}{G}\right]\right\}^{\frac{1}{2}}$$

$$\text{Phase}=\phi=-\tan\ \left\{\frac{\text{imaginary}\ G_{xy}}{\text{real}\ G_{xy}}\right\}$$

These values are smoothed by averaging one or more points and plotted as functions of frequency

ZUSAMMENFASSUNG

Der menschliche vestibulo-okulare Reflex (VOR) während aktiver Kopfbewegungen wurde durch Spek

tralanalyse untersucht um Verstärkungsfaktoren und Phasenwinkel für die natürlichsten Reizbedingungen zu extrahieren. Messungen wurden unter drei experimentellen Bedingungen vorgenommen 1) Kopfdrehungen im Dunkeln um Vergleichsdaten für die beliebteste Art der VOR Analyse zu liefern 2) Kopfdrehung während der Fixation eines stationären Lichtpunktes um natürliche kompensatorische Augenbewegungen zu quantifizieren und 3) Kopfdrehung während der Fixation eines Licht punktes der mit dem Kopf bewegt wurde als schnelle Methode zur Quantifikation der visuo-vestibulären Interaktion. Verstärkungsfaktoren für hochfrequente Kopfdrehungen wichen nicht signifikant vom Einheitswert ab Im Gegensatz zu früheren Beobachtungen während passiver Kopfbewegungen (Benson, 1970 Keller 1978) Mechanismen zur Erklärung dieser Diskrepanz werden diskutiert

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D W F Schwarz, M.D
Lab of Otoneurology
Dept of Otolaryngology
MSB room 7308
University of Toronto
Ontario
Canada

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EYE–HEAD COORDINATION DURING LATERAL GAZE IN NORMAL SUBJECTS

Takuya Uemura, Yasuko Arai and Chiga Shimazaki

From the Department of Otolaryngology Tokyo Women Medical College Shinjuku-ku Tokyo Japan

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Abstract. To elucidate the characteristics of eye–head coordination during lateral gaze eye head and gaze displacements to 10° 20° 30° 40° and 50° target presentations were analysed quantitatively in 10 normal subjects Head displacement was recorded without mechanical restraint by a newly devised apparatus containing two terrestrial magnetic sensors. Eye movements were recorded using d.c. electro-oculography through bitemporal leads. (1) As the target angle increased, the latency of eye movement increased while that of head movement decreased (2) The rise times of eye and head movements increased almost linearly with the target angle (3) The maximum velocity of eye movement averaged 297°/sec at 10° increased to 398°/sec at 20° and remained unchanged thereafter The maximum velocity of head movement increased linearly with the target angle (4) The head displacement at the end of eye saccade became relatively larger as the target angle increased, though the sum of eye and head displacements i.e. gaze agreed with the target angle (5) At the final resting position, the percentage of target angle attained by head displacement decreased from 85% at 10° to 62% at 50° However gaze displacement was exactly the same as the target angle with little dispersion for each angle of gaze shift, indicating that the head rotation detector is an accurate device for recording horizontal head movements under natural conditions

The unexpected appearance of a target in the visual field causes coordinated eye and head movements Initially the eyes move to fixate the target on the fovea. This saccade is followed by a head movement in the same direction During head movement, the eyes perform a countermovement and continue their target fixation In monkeys the vestibular system is primarily responsible for controlling the compensatory eye movement and nullifying target displacement from the fovea during this period of time (Dichgans et al 1973)

Little information is available on the eye–head coordination in humans This is at least in part due to the difficulty of recording head movements. In studying eye–head coordination it is essential to record the angular position of the head under the condition of maintaining normal head motility Although the potentiometer has been used to record head movements special equipment such as metal bellows was needed to absorb the rolling movement which frequently accompanied the head rotation in yaw (Barnes 1979) We have eliminated this problem by the method of utilizing a terrestrial magnetic sensor in which only a thin wire is connected to the subject (Uemura et al. 1976) Using this apparatus we now report on the characteristics of eye and head coordination during lateral gaze in normal subjects. These results will form the basis for studying the effect of labyrinthine lesions and other related abnormalities on the coordination of eye and head movements

MATERIALS AND METHODS

Horizontal head rotation was measured using a device containing two terrestrial magnetic sensors (Fig 1 A) The sensor was made utilizing the Hall effect by which an electric potential difference is developed between the edges of a metal strip carrying a longitudinal current when placed in a magnetic field perpendicular to the plane of the strip Terrestrial magnetism was used as a magnetic field It was reinforced by two pieces of Permalloy placed

tralanalyse untersucht, um Verstärkungsfaktoren und Phasenwinkel für die natürlichsten Reizbedingungen zu extrahieren Messungen wurden unter drei experimentellen Bedingungen vorgenommen 1) Kopfdrehungen im Dunkeln um Vergleichsdaten für die beliebteste Art der VOR Analyse zu liefern 2) Kopfdrehung während der Fixation eines stationären Lichtpunktes, um natürliche kompensatorische Augenbewegungen zu quantifizieren, und 3) Kopfdrehung während der Fixation eines Licht punktes der mit dem Kopf bewegt wurde als schnelle Methode zur Quantifikation der visuo-vestibulären Inter aktion Verstärkungsfaktoren für hochfrequente Kopfdrehungen wichen nicht signifikant vom Einheitswert ab im Gegensatz zu früheren Beobachtungen während passiver Kopfbewegungen (Benson 1970 Keller 1978) Mechanismen zur Erklärung dieser Diskrepanz werden diskutiert.

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D W F Schwarz M.D
Lab of Otoneurology
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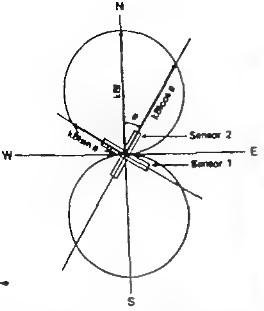


Fig 2 Principle of the head rotation detector When the device is placed in the terrestrial magnetic field so that the angle between sensor 2 and the direction of the magnetic field is θ the induced voltages of sensors 1 and 2 are $E_1 = kBI \sin\theta$ $E_2 = kBI \cos\theta$ where k the Hall coefficient B the magnetic flux density I the electric current density Thus, the angle of rotation can be detected by

$$\arctan(E_1/E_2) = \arctan(kBI \sin\theta / kBI \cos\theta) = \arctan(\tan\theta) = \theta$$

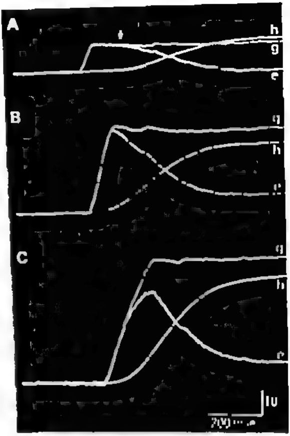


Fig 3 Original records of eye (e), head (h) and gaze (g) displacements to 10° (A) 30° (B) and 50° (C) target presentations The arrow indicates the start of compensatory eye movement which is synchronous with that of head movement.

40° and 50° right were made similar as in series 1

3) 10° right and 20° 40° 50° left

The right 20° lamp was used as the fixation lamp Target presentations at 10° right and 20° 40° 50° left were made as in series 1

Eye movements were calibrated before and after each examination. Calibration of the head movement was not necessary during the examinations because of the stable sensitivity of the terrestrial magnetic sensors

RESULTS

The five variables used for quantitative analysis were

1) latency between onset of target presentation and beginning of eye and head movements

2) rise time from beginning to end of the initial eye saccade and from beginning to end of the head movement

3) maximum velocity of eye and head movements estimated from the steepest tangent to the displacement curve

4) initial amplitude—eye and head displacement angles at the end of the eye saccade

5) final amplitude—eye and head displacement angles at final resting position

Before analysing all measured variables values showing a probability of less than 5% were rejected.

Fig 1 (A) The apparatus for recording horizontal head rotation (B) The helmet-mounted device containing two terrestrial magnetic sensors

at both sides of the Hall element (Fig 1B) Two terrestrial magnetic sensors were placed rectangular to one another they were mounted on the head by means of a light plastic helmet

The angular rotation around a fixed vertical axis can be detected from the ratio of the potential changes induced in each sensor as illustrated in Fig 2 The output voltage was proportional to the angular rotation within a range of ±50 with an error of less than 1% The total weight of the apparatus including the helmet was 450 g and only a thin wire made electrical connection to the head-mounted device

Horizontal eye movements were recorded using d c electro-oculography through bitemporal leads

The visual stimuli were red light-emitting diodes subtending the subject at a 0 14 visual angle The lamps were mounted at eye level in a 2 0 m radius semicircular panorama at 20 and 30° angles right and left with a center lamp

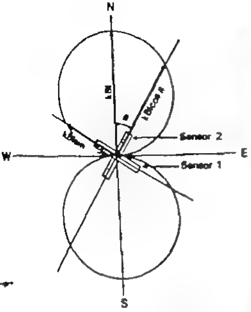
Target signals eye and head movements, and the sum of the eye and head movements (eye position in space or gaze) were displayed simultaneously by an ink writing oscillograph and stored on magnetic tape Data were analysed on a cathode-ray oscilloscope

Ten normal volunteers between the ages of 18 and 30 years participated in the present study They sat facing the fixation lamp and were instructed to move the head and eye to fixate on the target as it jumped from the center to one of the four lamps and back in 4 sec The examination was started after 1 min of dark adaptation

In order to examine lateral gaze displacement up to 50° in 10 steps by using the center and the four target lamps the following three series of examinations were undertaken

1) 20° 30° right and left
The center lamp was used as the fixation lamp The order of target presentation was randomized Each lamp was presented five times and each subject underwent a total of 20 trials

2) 10° left and 20° 40° 50° right
The subject sat facing the left 20° lamp which in this experiment represented the fixation lamp Target presentations at 10° left and 20°



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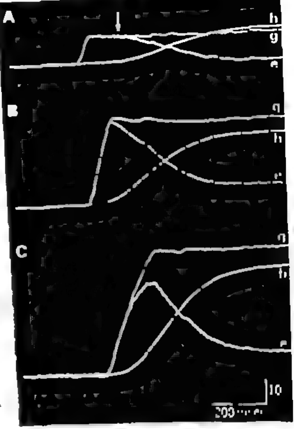
$= \arctan(\tan\theta) = \theta$



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Before analysing all measured variables values showing a probability of less than 5% were rejected

Table I *Latencies of eye and head movements (msec)*

L=left R=right

Target displacement	Eye		Head	
	Mean	S D	Mean	S D
10°L	245	41	389	72
20°L	244	34	350	63
30°L	261	35	344	54
40°L	268	47	313	33
50°L	304	53	350	57
10°R	273	53	390	65
20°R	264	44	353	60
30°R	278	40	353	47
40°R	279	41	338	48
50°R	314	70	359	63

Typical records of eye head and gaze displacements to 10° 30 and 50° target presentations are shown in Fig 3 In the 10° lateral gaze shift the eyes reach the target at the end of the saccade As the head begins to turn toward the target the eyes make a slow compensatory movement so that the gaze remains on target Eye and head movements were also well coordinated during lateral gaze shift of 30° and 50° although the amplitude of the eye saccade was smaller than that of the gaze

Latencies of eye and head movements

Latency data are presented in Table I The latencies of eye movement to target presentations on the left were significantly shorter than those to presentation on the right ($P<0.05$) The mean latencies of eye movement increased with the increase of the target angle (Fig. 4) On the other hand the latencies of head movement showed no difference between left and right target presentations Left and right responses were combined The mean latencies for 10 20° 30° 40° and 50° shifts were 389 (S D 65) 351(60) 349(47) 325(48) and 355(63) msec respectively decreasing with the increase in target angle except at 50° Thus in left gaze shifts the mean latency of head movement with respect to eye movement decreased from 144 msec at 10° to 57 msec at 40°

Rise times of head and eye movements

The left and right responses to each target angle were averaged as there was no significant difference between left and right target data. Although the mean rise times of eye saccade increased at about the same rate up to 50° for head movement the increase was limited to target angles smaller than 40° (Fig. 5)

Maximum velocities of eye and head movements

The mean maximum eye velocity for 10° shift was 292 /sec (S D 48) Velocities for target angles greater than 20° were of nearly the same value (398 to 410°/sec) On the other hand, maximum head velocities increased linearly with target angles (Fig 6) The relationship between target angle (X_t) and head velocity (Y) can be represented by

$$Y=1.39X_t+24.6$$

where the correlation coefficient is 0.65

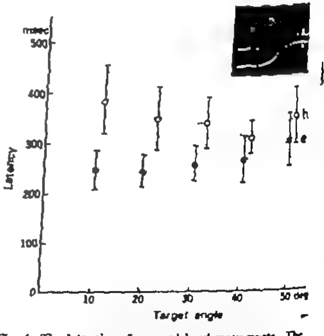

Fig 4 The latencies of eye and head movements The mean latencies of eye movement (*e*) for responses to left and those of head movement (*h*) for combined responses to left and right target presentation are plotted Solid and open circles in Figs 4 to 8 represent the mean values for eye and head movements respectively Vertical range bars show ±1 standard deviation.

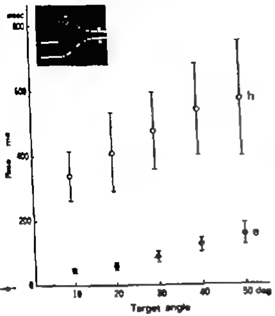

Fig 5 Rise times of eye (e) and head (h) movements

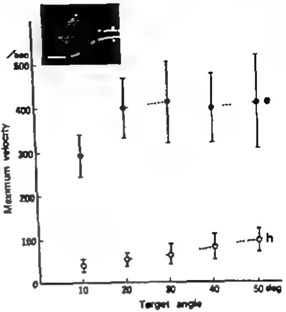

Fig 6 Maximum velocities of eye (e) and head (h) movements

The head displacement angle does not agree with the target angle as shown in Fig 8 later Thus, the relationship between head displacement (X_h) and head velocity (Y) is given in the following equation with a significantly higher correlation coefficient of 0 78 ($P<0.01$)

$$Y=2.41X_h+19.6$$

Initial amplitude

The initial amplitude of the eye movement for 10° and 20° gaze shifts was equal to the target angle (Fig 7) indicating that the head begins to move after the eyes have fixed the target on the fovea. For target angles greater than 30° eye displacement did not reach the target angle by the end of the eye saccade The initial amplitude of the eyes became relatively smaller for greater target angles. The mean eye displacement for a 50° shift was 85% of the target angle However the sum of eye and head displacements i e gaze continued to be equal to the target angle even for targets greater than 30°

Final amplitude

The displacement angles of the eyes and head at the final resting position are shown in Fig 8 At the final position the head scarcely reached

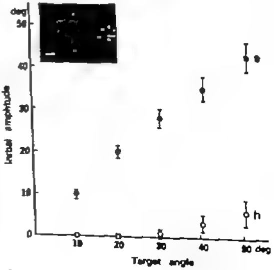

Fig 7 Initial amplitudes of eye (e) and head (h) movements.

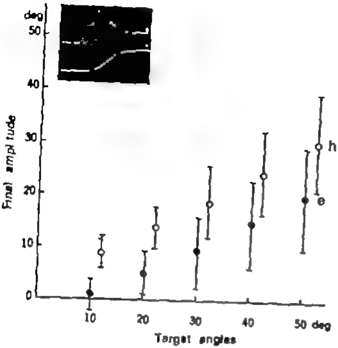

Fig 8 Final amplitudes of eye (*e*) and head (*h*) movements

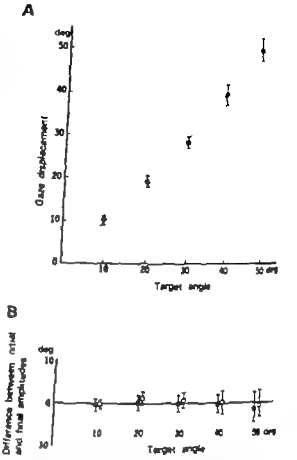

Fig 9 (A) The relationship between gaze displacement and target angle at the final resting position (B) The differences between the initial and final amplitudes of gaze Solid and open circles represent the mean values for right and left gazes respectively Vertical range bars indicate ±1 standard deviation.

the target angles even though the subjects were instructed to look at the target directly in front of the face The percentage of the target angle attained by head movement decreased with the increase in target angle it was 93% for a 10 64% for a 30 and 62% for a 50° shift However the percentage varied considerably among the different subjects as well as between trials performed with the same subject The coefficient of variation (standard deviation/mean) was 0 36 for 30° Nevertheless the gaze at the final position corresponded to the target angle with little dispersion (Fig 9A) The coefficient of variation was 0 05 for 30 The difference between the initial and final amplitudes as the magnitude of gaze was nearly zero for each target angle (Fig 9B) This proves that the target was fixed on the fovea at the end of the eye saccade

DISCUSSION

Fuchs (1971) who studied normal subjects with the head fixed reported that the latency of the eye saccade in response to a non predictable target increases with saccadic magnitude The gaze movements especially of large amplitude are mostly associated with head movements Gresty (1974) and Barnes (1979) using potentiometric recordings of head movements quantitatively analysed eye and head movements of normal subjects during free head tracking The mean latencies of eye movement increased from 243 msec at 15° to 383 msec at 75 (Barnes 1979) Our study revealed a similar tendency—the mean latencies increased from 245 msec at 10° to 304 msec at 50 However the mean latencies of head movement decreased from 389 msec at 10 to 325 msec at 40° although the latency rose to 355 msec at 50° This finding is contrary to that of Barnes (1979) who reported a positive correlation between latency and

target angle. When comparing the mean latency obtained by Barnes (1979) for 15° with our 10°, there was a delay of approximately 100 msec in our data. On the other hand, his value for 45° and ours for 40° were similar. The onset of the head movement for a 10° shift could also be measured by the onset of compensatory eye movement, because the eyes almost always reached the target before the head movement started (Fig. 3, arrow). That the latencies thus obtained also showed such a delay denies the presence of experimental error in the head movement recording technique associated with slippage of the head within the helmet, etc.

The characteristics of saccadic eye movements such as duration, termed here the rise time, and maximum velocity are very similar to those reported in normal subjects with the head fixed (Becker & Fuchs, 1969).

The approximately linear relationship between maximum head velocity and target angle reported by Gresty (1974) and Barnes (1979) was also observed in our data. However, the maximum head velocity was more closely related to the angle of head displacement, which was actually performed at each trial. Thus, head movement does not appear to be well correlated with retinal error information.

At target angles of less than 30°, the target can be caught by the eye saccade alone. When the mean values of eye and head displacement at the end of the eye saccade were added at the larger target angles, the sum, i.e. gaze displacement, was 28.0+0.9=28.9° at 30°, 35.2+3.7=38.9° at 40°, and 42.7+6.5=49.2° at 50°. These values corresponded to more than 95% of each target angle, indicating that the eye saccade fixed the target on the fovea with the associated head movement and that thereafter the compensatory eye movement started in the opposite direction. That the target was caught on the fovea before compensatory eye movement began was also confirmed by the fact that, when initial and final gaze amplitudes were compared in order to eliminate errors in measurement, the mean values of their differences were nearly zero at all target angles (Fig. 9B).

Although eye and head displacements at the final resting position showed large variations among different subjects and also between trials with the same subject, the gaze displacement at each eye–head movement agreed very well with the target angle. These results were as expected, since normal subjects must fix the target on the fovea at the end of eye and head movements. In other words, this confirms the accuracy of the present recording techniques used for eye and head movements. The recording methods we used can record eye and head movements within a gaze displacement of 50° with satisfactory accuracy, even if there are experimental artifacts such as changes in the sensitivity of the terrestrial magnetic sensors due to the subject's head tilt or changes in electro-oculography calibration due to variations in the corneoretinal potential.

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We thank Dr B. Cohen for critically reading the manuscript and Kaoru Imaoka and Masao Yamamoto for technical assistance.

ZUSAMMENFASSUNG

Um die Merkmale der Auge-Kopf Koordination während seitlichen Blicks zu erläutern, wurden die Augen-, Kopf- und Blickverschiebungen von [illegible] normalen Subjekten bei 10°, 20°, 30°, 40° und 50° Zielvorgaben quantitativ analysiert. Die Kopfverschiebung wurde ohne mechanische Zurückhaltung durch ein neu entwickeltes Gerät mit zwei irdischen, magnetischen Sensoren aufgezeichnet. Augenbewegungen wurden durch DC Elektrookulographie mittels bitemporaler Leitungen aufgezeichnet. 1. Bei größerem Zielwinkel erhöhte sich die Latenz der Augenbewegung, während die der Kopfbewegung kleiner wurde. 2. Die Amplituden von Augen- und Kopfbewegungen nahmen fast linear zum Zielwinkel zu. 3. Die Spitzengeschwindigkeit der Augenbewegung kam bei 10° im Durchschnitt auf 292°/sec, nahm bei 20° auf 398°/sec zu und blieb danach unverändert. Die Spitzengeschwindigkeit der Kopfbewegung nahm linear zum Zielwinkel zu. 4. Die Kopfverschiebung am Ende der Augensakkade wurde mit zunehmendem Zielwinkel relativ größer. Hingegen stimmte die Summe von Augen- und Kopfverschiebungen, d. h. des Blicks, mit dem Zielwinkel überein. 5. Bei der schließlichen Ruhestellung nahm der durch Kopfverschiebung erlangte Prozentsatz des Zielwinkels von 93% bei 10° auf 62% bei 50° ab. Hingegen war die

Blickveränderung genau dieselbe wie der Zielwinkel was auf die Prazision des Kopfrotationsdetektors als Instrument zur genauen Aufzeichnung horizontaler Kopfbewegungen unter natürlichen Bedingungen hinweist.

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T Uemura
Department of Otolaryngology
Tokyo Women's Medical College
10 Kawado-cho
Shinjuku-ku Tokyo 162
Japan

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QUANTITATIVE STUDY OF HUMAN SCARPA'S GANGLION AND VESTIBULAR SENSORY EPITHELIA

Ernst Richter

From the Department of Otolaryngology, Massachusetts Eye and Ear Infirmary and Harvard Medical School, Boston, Massachusetts, USA

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Abstract. Methods for counting vestibular ganglion cells and determining the densities of hair cells and intraepithelial basophilic inclusions (IBI) in samples of cross-sectioned vestibular sensory epithelia are described. Data obtained by means of these methods in vestibular sensory epithelia and Scarpa's ganglia in individual temporal bones from subjects at different ages are presented. Both vestibular hair cells and nerve cells in Scarpa's ganglia are found numerically reduced in ears of aged individuals. Changes in the vestibular sensory epithelia appear to precede those seen in Scarpa's ganglion. The incidence of intraepithelial basophilic inclusions correlates with degeneration in the respective vestibular sensory epithelia. There are no striking differences in hair cell densities of the different vestibular sense organs of the same ear and from subjects at about the same age.

In order to evaluate the pathological changes in the peripheral vestibular system seen in a light-microscope examination of temporal bones, it has first to be established what can be considered as a normal vestibular sense organ or nerve at a given age. Such normative data should include cell counts for the sensory cells and associated nerve cells.

Quantitative analyses of hair cell populations in the human vestibular sensory epithelia were made by Rosenhall (1972, 1973). Using the surface preparation technique (Retzius, 1884; Engström et al., 1962; Lindeman, 1969*a*) he measured the areas of the different vestibular sensory epithelia and sampled hair cell density to estimate total hair cell populations.

Numerical data on primary vestibular neurons in man are available from studies by Rasmussen (1940) who counted extramedullary nerve fibers, by Bergström (1972, 1973) who counted myelinated fibers peripheral to the ganglion, and by Naufal & Schuknecht (1972) who counted ganglion cells in temporal bone sections.

Engström et al. (1974) concluded that vestibular sensory cells became reduced in number at about 40 years of age and that this reduction was paralleled by a reduction in the number of vestibular neurons as reported by Bergström (1972, 1973). These changes seemed to be preceded by more subtle degeneration in the sense organs observed under the light and electron microscopes by Rosenhall & Rubin (1975). One commonly described pathological change is the appearance of cysts and intraepithelial spaces in various epithelia of the vestibular sense organs (Mayer, 1907; Alexander, 1924; Kolmer, 1927; Werner, 1933; Borghesan, 1963; Lindeman, 1969*b*). Rosenhall (1974) studied epithelial cysts, which he had found in the cristae of vertical semicircular canals in ears from aged individuals only.

One major limitation of the previous quantitative data is that sensory cell and nerve cell counts have not been performed on the same ear. With serially sectioned temporal bones (rather than surface preparations) both types of counts can be performed.

The purpose of the present study was to provide counts of sensory cells, nerve cells and intraepithelial inclusions as seen in tem-

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Blickveränderung genau dieselbe wie der Zielwinkel was auf die Präzision des Kopfrotationsdetektors als Instrument zur genauen Aufzeichnung horizontaler Kopfbewegungen unter natürlichen Bedingungen hinweist

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T Uemura
Department of Otolaryngology
Tokyo Women's Medical College
10 Kawado-cho
Shinjuku-ku Tokyo 162
Japan

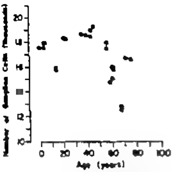

Fig. 1. The total numbers of vestibular ganglion cells (corrected for split nucleoli) as function of age

$$N_i = n_i \frac{t}{t+d}$$

where N = the estimated number of neurons n_i = counted number t = the thickness of sections d = the diameter of the nucleoli.

The thickness of the first and last sections containing ganglion cells was measured in each temporal bone by means of the micrometer on the fine-focus control and was within 10% of 20 µm. Nucleoli from different ganglia were measured by means of a drawing tube attached to the microscope. The average diameter was found to be 4 µm. Thus to estimate the total number of ganglion cells in any one case the total number of cells counted in every tenth section was multiplied by 10 and by the correction factor 0.83

4. Density of vestibular hair cells and intraepithelial basophilic inclusions (IBI)

Hair cell density was estimated by counting the number of hair-cell nuclei appearing in one cross-section through each sensory epithelium and dividing that count by the estimated surface area of the epithelium in that section. Surface area was estimated by multiplying the section thickness by the distance along the endolymphatic surface of the epithelium seen in that section. Density was expressed per 0.01 mm². Samples in the cristae were at least 0.008 mm² and in the maculae 0.0012 mm²

bone

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RECONSTRUCTION OF SCARPA'S GANGLION

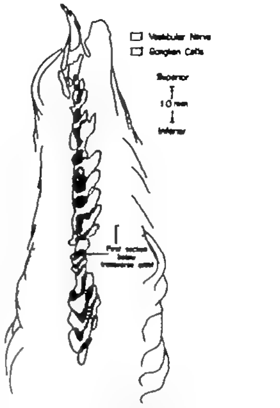

Fig. 2. Profiles of the vestibular nerve and Scarpa ganglion drawn by means of microprojector from every 10th horizontal section. Most superior is the section on top. Superimposed areas in adjacent drawings are more darkly shaded

first cross section seen when scanning the set of slides from superior to inferior. Cell counts could not be made in some cases because of unfavorable cutting angle or postmortem autolysis

IBIs are defined by their size (the maximum being as large as about three hair cells) by their basophilic content and by their location in the sensory epithelia. IBIs were counted in the same samples as the hair cells.

RESULTS

1. Counts of neurons in Scarpa's ganglion

As can be seen in Table I the superior division contained more ganglion cells than the inferior division in all but one case (5-year-old right

Table I *Cell counts in Scarpa s ganglion*

Counts corrected for *split nucleoli* in the superior division in the inferior division and the sum of both in the right column

Age	Side	Sup div	Inf div	Total
9 w	R	11 07[illegible]	[illegible] 623	17 695
9 w	L	9 294	6 374	15 668
4	R	10 715	7 337	18 052
4	L	12 026	5 594	17 620
5	R	8 931	9 088	18 019
5	L	10 225	8 998	19 222
11	R	11 470	8 765	20 235
14	R	10 341	5 727	16 068
14	L	8 790	7 071	15 861
16	L	12 832	7 295	[illegible]0 127
16	R	11 437	7 987	[illegible] 9[illegible]4
16	L	11 952	8 773	20 725
21	R	11 197	7 295	18 49[illegible]
21	L	9 985	7 287	17 27[illegible]
23	R	12 375	6 009	[illegible] 384
23	L	10 765	5 494	16 259
30	R	11 355	5 801	17 156
35	R	9 578	6 698	16 [illegible]76
35	I	10 [illegible]4[illegible]	7 986	17 728
36	R	1[illegible] 325	8 [illegible]76	20 600
36	L	10 823	7 926	18 749
41	R	10 765	7 860	18 6[illegible]5
41	L	10 283	7 752	[illegible] 035
44	R	12 284	6 747	19 031
44	L	11 3[illegible]1	7 [illegible]71	18 592
46	R	1[illegible] 226	7 104	19 330
48	R	9 155	7 234	16 891
53	L	8 648	6 864	15 512
57	R	11 487	7 113	[illegible] 600
57	L	10 176	9 179	19 355
57	R	9 088	8 956	18 044
57	L	8 856	8 657	17 513
58	R	7 273	4 936	12 [illegible]09
58	R	7 080	5 660	12 740
59	R	7 569	7 [illegible]79	14 848
61	R	[illegible] 906	7 1[illegible]1	16 027
61	L	8 781	6 391	15 17[illegible]
62	R	11 379	6 640	18 019
62	L	10 283	5 669	15 95[illegible]
67	R	7 818	6 964	14 782
68	R	7 171	5 395	1[illegible] 566
68	L	6 814	6 009	12 823
72	R	8 19[illegible]	7 030	15 [illegible]
72	L	8 889	7 885	16 774
73	R	8 698	4 764	13 462
73	L	7 843	4 897	1[illegible] 740
76	R	10 582	6 105	16 687
76	L	8 964	6 947	15 911
79	R	7 146	5 660	12 806
85	R	9 039	5 353	14 39[illegible]
85	L	6 963	5 287	1[illegible] 250
87	R	6 507	5 303	11 810
89	L	8 640	5 196	13 836
91	R	11 736	5 685	17 4[illegible]1

poral bones of human subjects of different ages without known disorders that could have affected the vestibular system Such data should serve as a baseline against which to compare similar counts taken from subjects with known vestibular disorders In addition the relationship between the numbers of various cell types in "normal ears could be investigated

MATERIALS AND METHODS

1 *Selection of temporal bones*

Cell counts were made of the vestibular ganglion in 54 human temporal bones and the vestibular hair cell and inclusion body densities were estimated in 30 of these None of these 54 temporal bones were from subjects who had diseases or treatments that might have affected the vestibular sensory epithelia or nerves (e g Meniere s disease diabetes mellitus cytostatic or ototoxic medications radiation of the head infection neoplasms of the temporal bone)

2 *Processing of temporal bones*

The temporal bones were removed between and 22 hours postmortem fixed in Heidenhain–Susa solution decalcified and embedded in celloidin Specimens were sectioned in the horizontal plane at 20 μm thickness Every tenth section was stained with hematoxylin and eosin and mounted for light microscopic examination (Schuknecht 1971)

3 *Ganglion cell counts*

The nucleoli of nerve cells in Scarpa s ganglion were counted in every tenth section under the light microscope using a magnification of 450× A manual cell counter and an ocular grid were used The entire ganglion has an hourglass shape the narrow region separates the inferior from the superior division The plane of section used was perpendicular to the long axis of the hourglass Thus the boundary between the two divisions was arbitrarily set at the level showing the fewest ganglion cells.

To correct for the errors introduced by double counting of nucleoli split between two sections the formula of Abercrombie (cited by Konigsmark 1970) was used

Fig. 4 (*a*) Detail of lateral crista with an intraepithelial basophilic inclusion (*arrow*). Part of the detached cupula can be seen above the epithelium. (*b*) Cross sectioned macula utriculi containing 2 intraepithelial basophilic inclusions (*arrow*).

utriculi below. In only a few instances could nerve endings be identified under the inclusions or were hair cells found riding on top of them. Both hair cells and inclusion bodies were evenly distributed throughout the regions evaluated.

Using the same technique, counts were done in subsequent sections and calculated densities differed less than 3% from original values.

Larger cysts filled with basophilic material were also seen. They were sometimes multiple and had an epithelial lining. In the cristae they were found at the peripheral borders of the sensory epithelium on the top or sometimes in the transitional epithelium only. They were more frequently seen in ears of older individuals. Multiple cystic spaces in the posterior canal crista of a 57 year-old subject are shown in Fig. 5. This type of cyst was not included in the counts of inclusion bodies.

Fig. 6*a* shows a plot of hair cell density

Fig 3 Detail of normal sensory epithelium of the most superior cross section of a macula sacculi. Each of the five arrows points to a hair cell nucleus.

ear) In this case the separation of the two divisions had been difficult to determine The mean counts in the inferior division were roughly two-thirds those of the superior division

The relation between age and the total number of ganglion cells is seen in Fig 1 Around age 60 there is a significant drop in the average number of ganglion cells

A reconstruction of the three-dimensional form of Scarpa s ganglion is shown in Fig 2 The hourglass shape is evident The arbitrary dividing line between superior and inferior division is marked by an arrow The vertical extent of the ganglia ranged from 140 to 220 sections i e 2 8 to 4 4 mm There was no apparent correlation between the extent of the ganglia and the age of the subjects Much of this variability could be due to differences in cutting angle

2 *Density of hair cells and intraepithelial basophilic inclusions (IBI)*

A cross section through the macula sacculi (Fig 3) shows the relative location of hair cells and basal supporting cells IBIs are demonstrated in Fig 4 A single inclusion is shown in a lateral crista and two IBIs in a macula

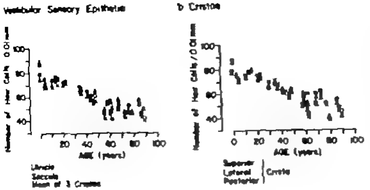

Fig. 6. Density of vestibular hair cells as a function of age. In panel A, values for the utricle and the saccule and mean of the three crista are plotted for each case. In B, the individual values of each crista are shown.

populations is shown. It appears that when hair-cell density was low, ganglion cell counts in the same ear were highly variable. However, if the hair-cell density was high, ganglion cell counts were always high.

DISCUSSION

1. *Neuronal counts*

Counts of primary vestibular neurons have been obtained at different levels. The numbers obtained by counting ganglion cell bodies (as in the present study by Naufal & Schuknecht 1972) are about the same as those found by counting nerve fibers central (Rasmussen 1940) or peripheral (Bergström 1972, 1973) to Scarpa's ganglion. Nerve counts from aged individuals were typically lower, and the reduction was about the same in all these studies. It is interesting that counts of neuronal populations in brainstem nuclei do not appear to decline with age (Konigsmark 1969, Konigsmark & Murphy 1972). In our data, the mean count for the inferior divisions was approximately two-thirds that of the superior divisions. These data are in agreement with Bergström's (1972) findings; however, different distribution was obtained by Naufal & Schuknecht (1972). They had arbitrarily chosen the appearance of the saccular nerve as the point of division. This reference point would not necessarily occur in the same section as the constriction in the ganglion, which was used as the point of division in this study. The total cell counts from Naufal & Schuknecht were in the range of our uncorrected numbers with the exception of a lower count in a pathological case. Reduction of counts in aged individuals in the present study was about the same for the superior and inferior

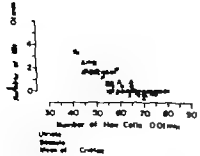

Fig. 7. Relationship between densities of vestibular hair cells and IBI densities.

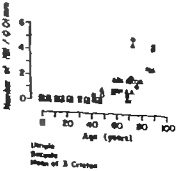

Fig. 8. Density of IBI as a function of age.

Fig 5 Posterior crista with subepithelial cystic inclusions containing basophilic staining material (arrow)

per 0 01 mm² as a *function* of the subject s age The general trends are for a decreased cell density with increasing age and a marked increase in the spread of densities at ages above 50 years There are only slight differences between sensory structures in the same ear However there were in some cases significant differences between the two ears in the same individual It is interesting that the exceptionally high hair cell densities in the youngest subject were not paired with exceptionally high ganglion counts (Table I) The mean degeneration was slightly more pronounced in the saccule and cristae than in the utricle The variability is high for ages over 55 years but even the highest values never reached values comparable to those of ears under 40 years Fig 6*b* shows the individual values of the three cristae

As shown in Fig 7 and 8 the density of IBIs is inversely proportional to hair cell density and positively correlated with the subject s age

3 Ganglion cell counts as a function of IBI and hair cell densities

In Fig 9*a* and *b* the relation of changes in the sensory epithelial to changes in the neuronal

epithelia in aged individuals were all below the range obtained in those under 30 years

The incidence and densities of inclusion bodies increased with aging and was correlated with degeneration of sense organs. In some cases a hair cell had been found on top of an inclusion body. Usually no hair cells were seen within these inclusions. It is not unlikely that complete degeneration of a hair cell leaves an inclusion body within the nerve ending in the sensory epithelium but such bodies could also represent degenerated nerve endings, as shown in guinea pigs by Watanuki & Kagayama (1973)

3 *Relationship of hair cell densities to neuronal counts*

In individuals more than 35 years old hair cell densities are low but a normal ganglion cell population could be counted. Only above age 55 was loss of nerve cells seen. Neuronal degeneration was never seen in cases with normal hair cell densities. Such 'primary' nerve degeneration might be found in young individuals with vestibular schwannoma. The findings of Konigsmark & Murphy (1972) seem to preclude a numerical reduction of brainstem nuclei in aging. Thus the frequently observed dysequilibrium of aging could mainly be a problem of the peripheral vestibular system. From the present findings different anatomical substrates are proposed for dysequilibrium of aging: sensory degeneration appearing early and sensory-neural degeneration appearing not before age 55

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ZUSAMMENFASSUNG

Methoden, um primäre vestibuläre Neurone zu zählen und die Dichte von Haarzellen und intraepithelialen basophilen Einschlüssen in Proben von Querschnitten vestibulärer Sinnesepithelien zu bestimmen, werden beschrieben. Die Ergebnisse quantitativer Untersuchungen vestibulärer Sinnesepithelien und Scarpas Ganglion in Felsenbeinen von Individuen verschiedenen Alters werden berichtet. Sowohl die Anzahl primärer vestibulärer Neurone als auch die Dichte von Haarzellen ist in den Felsenbeinen von alten Personen vermindert. Diese Veränderungen scheinen in den vestibulären Sinnesepithelien früher aufzutreten als in Scarpas Ganglion. Die Dichte der intraepithelialen basophilen Einschlüsse korreliert mit dem Ausmaß der Degeneration in den entsprechenden Sinnesepithelien. Die Haarzelldichte variiert nur wenig zwischen verschiedenen vestibulären Sinnesorganen im selben Innenohr oder in Felsenbeinen von Individuen, die etwa gleich alt sind.

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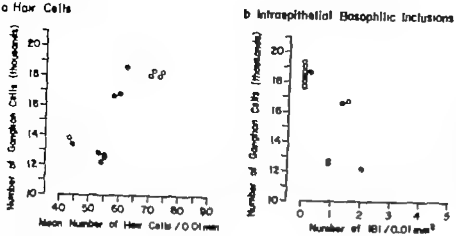

Fig 9a b Total number of ganglion cells (corrected for split nucleoli) as a function of hair cell density (panel A) and as a function of IBI density (panel B). Hair cell densities are averaged across all epithelia in each case

division No tendency was seen for one division to show greater loss than the other one at any age

However it is interesting that the nerve cell counts did not decrease gradually but that there was a steep decrease at about age 60 If the numbers of neurons counted in the present study were plotted as a function of the year of birth of the individuals all low counts would derive from subjects born before 1920 An event in common to many individuals or different events before 1920 could have induced a reduction of neurons at one time Thus the values obtained here should only serve for comparison to values in pathological cases if a population not only of about the same age but also birth-year is selected

2 *Hair cell densities and inclusion bodies (IBI)*

In the present study lower densities of hair cells were found for subjects more than 30 years old Selective degeneration of one sense organ only with normal counts in the remainder of the vestibular epithelia has not been observed in the cases presented This decrease seemed to precede that for the nerve cells in the same individuals None of the individuals more than 50 years old had densities within the range of those below 30 years

Mean hair cell densities at all ages were lower than those reported by Rosenhall (1972 1973) The reason for this discrepancy is unclear The percentage decrease in hair cell densities which we found in the cristae of subjects above 70 years was about the same as that reported by Rosenhall (1973) however in our maculae the percentage decrease was larger Rosenhall reports that degeneration was more pronounced in the striolae Our counts probably sampled from both striolae and peripheral regions of the maculae These results are in conflict with those of Engström et al (1974) and Rosenhall & Rubin (1975) who suggest that loss of hair cells is paralleled by a reduction of neurons It is difficult to determine from their data when the decrease actually occurs since the samples are small or have gaps in the middle age range The numerical decrease of hair cell densities found in ears of old subjects is in contrast to the conclusions of some previous studies not based on quantitative analyses (von Fieandt & Saxén, 1937 Schuknecht 1955 Jorgensen 1961 1964 Hansen & Reske-Nielsen 1964 Reske Nielsen 1964)

It has been reported that in ears of aged individuals the utricle shows less degeneration than the saccule (Johnsson 1971 Johnsson & Hawkins 1972) Schuknecht (1965) has suggested that cochleo-saccular degeneration may underlie one type of presbycusis Characteristic of this condition were changes in the saccule and cochlea, but no changes in the rest of the vestibular labyrinth were seen Cases of presbycusis are probably included in the present study But values of the different sensory

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PERILYMPHATIC PRESSURE IN THE CAT

Description of a New Method for Study of Inner Ear Hydrodynamics

B Carlborg, O Densert and J Stagg[3]

From the [1]Department of Otolaryngology, Malmö General Hospital, (University of Lund), Malmö, Department of Otolaryngology, Halmstad General Hospital, Halmstad and [3]Department of Experimental Research, Malmö General Hospital (University of Lund), Malmö, Sweden

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Abstract. Continuous recordings of the perilymphatic (P_p), cerebrospinal fluid (P_{csf}), central venous (P_{cv}) and arterial (P_a) pressures have been performed on anaesthetized cats. The perilymphatic space was reached by an extra-aural approach that leaves the ear canal and middle ear intact. A conical canal was drilled into the temporal bone down to the vestibule. The position of the canal was later confirmed histologically. A small, threaded, metallic cannula was screwed into the bone. For pressure measurements Millar microtip transducers were used, thus giving minimum measurement volume displacement. The mean P_p and P_{csf} were found to be equal, whereas the maximum pressure during expiration was significantly higher in the CSF. The mean P_p and P_{csf} were not affected by the given anaesthetics. Ligation of the external jugular veins had minor and temporary effect on the P_p and P_{csf}. The method is considered to be suitable for studies on the inner ear hydrodynamics as well as for audiophysiological measurements with an intact middle ear transmission system.

Experimental studies on the inner ear hydrodynamics are essential for a better understanding of hearing physiology and especially conditions associated with changes in inner ear pressure (e.g. Menieres disease, Alternobaric vertigo and perilymphatic fistulas).

Menieres disease is morphologically characterized by an endolymphatic hydrops, which has been considered to be associated with raised endolymphatic pressure (Hallpike & Cairns, 1938, Altmann & Kornfeld, 1965). Changes in ambient pressure, e.g. when diving and flying, may in some cases lead to transient attacks of vertigo, the socalled Alternobaric vertigo (Mellvill Jones, 1957; Tjernström, 1974). Other people may under similar conditions encounter sudden sensorineural hearing loss and even perilymphatic fistulas (Goodhill et al., 1973; Nedzelski & Barber, 1976; Freeman, 1978; Simmons, 1978).

The inner ear pressure has been studied experimentally for a long time. Even before actual recording of the inner ear pressure was carried out, Weber Liel (1879), Politzer (1901) and Szász (1926) made extensive visual observations through a fistula to the inner ear. Along with the gradual improvement in methods and technical equipment, more detailed and reliable studies have been made of the inner ear pressure and its relations to changes in intracranial pressure (Hughson, 1932; Kobrak, 1933; Kerth & Allen, 1963; Martinez, 1968; Beentjes, 1970; Angelborg & Ågerup, 1975), body position (Martinez, 1968; Parker, 1977) and blood pressure (Martinez, 1968).

However, very little is known about the way in which the inner ear is affected by changes in ambient pressure, or to what extent variations in metabolic, vascular or pharmacological conditions affect the inner ear pressure.

The purpose of this paper is to present a new method for the study of these questions. Such a method would favour the following requirements: 1) An extra-aural approach to the inner ear, i.e. an approach that keeps the ear canal and middle ear intact; 2) minimal volume displacement in the pressure measurement system; 3) control of basic physiological factors; 4) continuous recording for several hours.

MATERIAL AND METHODS

The present study was carried out on 20 healthy mongrel cats with weights of 2.5–3.5

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Ernst Richter
Massachusetts Eye and Ear Infirmary
243 Charles Street
Boston Mass 02114 USA

Fig 2 Histological picture showing the temporal bone A canal ( ) drilled via the the extra-aural approach into the vestibule (*b*). No bleeding or damage was seen in the inner ear The stapes ( ) stapes (*d*), facial nerve (*e*), and semicircular canal (*f*).

varied as desired via the other one The inner diameter of the cannulas was 1 mm and the total length [illegible] mm

When calculating the mean perilymphatic and CSF pressures account was taken for the difference between respiratory and pulse pressure variations Therefore the integrated mean values were used and calculated according to the following formula (Hallén 1953)

$$P = \frac{1}{T}\int (p)\,dt$$

P_m = mean pressure value T = time (one respiratory cycle) $(p)dt$ = instant pressure value

The central venous pressure (P_{CV}) was recorded via a polyethylene tube (PE 190) inserted in a central direction in the external jugular vein. To investigate possible effects on the P by ligation of the external jugular vein this was first carried out on one side and later simultaneously on both sides whilst recording the P_P.

The arterial blood pressure (P_A) was continuously measured in the femoral artery via a polyethylene tube (PE 190)

Since the P was of major interest in our experiments the vestibule of the inner ear was selected as reference zero level for all the pres

Fig 1 View of the temporal bone showing the extra-aural approach to the inner ear A cannula (*a*) is screwed into a canal drilled in a fronto-medio-caudal direction cranially to the mastoid process (*b*). The malleus (*c*), the tympanic bulla (*d*)

kg Pentobarbital was used for intravenous anesthesia with an induction dose of 30 mg/kg body weight and supplementary injections of 10 mg/kg In order to have full control of blood gas pressure levels the animals were tracheotomized and ventilated with a respirator Arterial blood gas analysis was repeatedly carried out (ABL 1 Acid Base Laboratory Radiometer Denmark) The animals were supplied intravenously with 5 5% glucose approximately 15 ml/hr The body temperature was continuously recorded rectally (TE3 Lab Instruments) and was kept at 36–38°C with the help of an electric blanket

The perilymphatic pressure (P_P) was measured via an extra-aural approach that leaves the tympanic cavity and the ear canal intact for audiophysiological measurements The temporal bone above the osseus external ear canal was exposed Using an operating microscope a conical canal was drilled in a fronto-caudo-medial direction cranial to the mastoid process (Fig 1) Without bleeding or damage to other structures the bony vestibular wall was perforated (Fig 2) Clear perilymph was then seen to run immediately into the drilled canal A threaded metallic cannula (I D 0 8 mm length 17 mm) filled with artificial cerebrospinal fluid (Pappenheimer et al 1962) was firmly screwed into the canal without penetration of the vestibule The cannula is shown in Fig 1 An additional seal was effected by applying epoxy resin around the cannula

The cerebrospinal fluid pressure (P_{CSF}) was recorded in one of the lateral ventricles A craniotomy was performed through the parietal bone on both sides of the sagittal suture Sharpened metallic cannulas were inserted into both ventricles By this method the P_{CSF} could be recorded via one of the cannulas and

fixed by a steel bar drilled through the upper canine teeth according to a method described by Henriksson et al (1961)

Histological studies were performed later in order to verify the location of the canal drilled into the inner ear When the experiment was concluded the animal was sacrificed with an overdose of pentobarbital The temporal bone was removed and fixed in 4% buffered formalin solution. It was then decalcified in formic acid dehydrated in alcohol embedded in paraffin and sectioned for light microscopy

Technical equipment

For measurement of P_p, P_{csf} and P_{cv} Millar microtip transducers PC 360 6F were used. They consist of a 1?0 cm long catheter with a pressure-sensor mounted at the distal tip (diameter 2.0 mm) The pressure sensor is a semiconducter gauge which produces an electrical output signal which varies in direct proportion to the magnitude of the sensed pressure The volume displacement of $1\times10^{-}$ mm³/13.3 kPa and the frequency response of 0–20 kHz renders the microtip transducer suitable even for high frequency pressure measurements (Millar Instruments Inc Houston Texas USA)

The microtip transducers were coupled to the metallic cannulas by means of similar rigid plastic adapters (Fig 2) These were filled with artificial CSF fluid The transducers were inserted into the adapters with great care during continuous recording of the pressure Increases of P above 3 4 kPa were not seen

The total volume of the cannulas and adaptors for measurement of the P and the P_{csf} were approximately 20 mm³ and 25 mm³ respectively

For measurement of the P a conventional pressure transducer EMT 34 (Siemens Elema, Stockholm Sweden) was used coupled to an EMT 311 amplifier (Siemens Elema)

Calibration of all transducers was performed against a known water column before and after each experiment The transducer zero levels were checked at regular intervals during the experiment Care was taken to ensure that variations in illumination or temperature did not cause inaccuracies

The output signals from the microtip transducers were processed in a specially designed amplifier with built-in calibration. In this amplifier encapsulated isolater units were used to isolate completely the transducers from earth and main supply They utilized high frequency modulation to transfer the measurement signals and transducer power across an insulating transformer To boost the measurement signal well above the noise level in the isolater a pre amplifier with ×20 gain is incorporated in the insulated circuit Another amplifier after the isolater brings the signal up to a level suitable for driving a recording device The output signals from the amplifier were simultaneously recorded on an ink-jet recorder (Mingograph 800 Siemens Elema) and a four-channel instrumentation tape recorder (Tandberg Series 115 Norway) to allow replay for more detailed analysis In Fig 4 the instrumental set up is shown schematically

RESULTS

Thirteen out of 20 experiments were considered successful Histological examinations carried out in seven of the successful experiments confirmed that the canal drilled into the inner ear had perforated the vestibule without damage to other structures (Fig 2) The reason for failure of some experiments was inaccurate direction of the drilled canal with penetration to the middle ear facial nerve canal or a semicircular canal The pressures were recorded continuously for as long as was considered desirable (5–12 h) No major pressure changes were seen as long as ventilation circulation and temperature were kept stable There were no problems with leakage around the cannulas or adapters

During normal conditions the maximum variations between expiration and the inspiration were found to lie within the range of 0 15–0.74 kPa for P_{csf} and 0 06–0.37 kPa for P_p. The method of paired comparisons with the Stu-

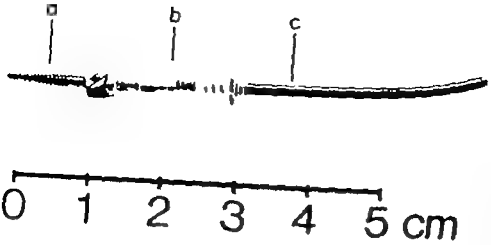
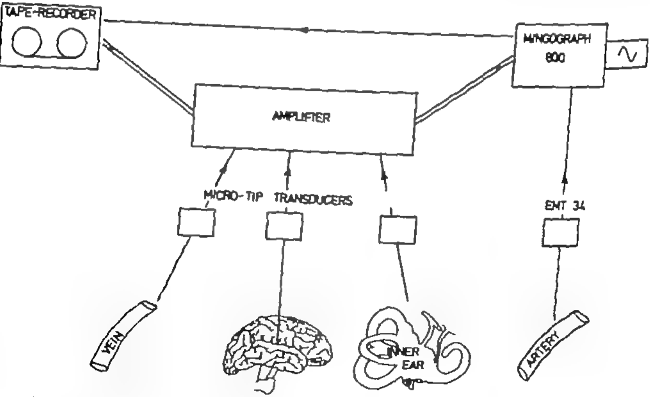


Fig 3 The inner ear ca-
nula a threaded metalic
cannula ( ) surmounted b
a rigid plastic adapter (b)
A microtip pressure trans-
ducer (*c*) is inserted throu
a rubber "O" ring into the
adaptor

sures The exact level was identified in each experiment via the drilled canal and thus correction for *hydrostatic* pressure differences could be *made for* all the transducers

The cat was positioned with its back upwards on a platform Rotation of the platforn was possible about a transverse axis througl the zero level The head of the animal was



4 Experimental set-up for continuous measurement
the perilymphatic cerebrospinal fluid enous and
erial pressures.

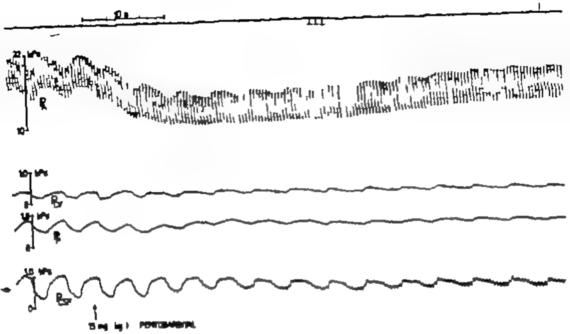


Fig. 6. A registration example showing the effect of first intravenous injection of pentobarbital 15 mg/kg. A large transient fall in P_p is observed. Due to the respiratory depression there is reduction of the maximum P_p and P_{csf} levels, whereas the mean pressure levels are only slightly affected.

the formula described above. No significant difference ($p>0.05$) between the mean P_p and mean P_{csf} was found (Table I). The normal mean P_p was between 0.34 and 1.77 kPa. However, within each animal the mean P_p showed only small variations during normal physiological conditions. Calculation of an integrated mean value from the registration examples involved a possible error of 0.03 kPa. The maximum P_p pressure lags behind the maximum P_{csf} by 0.2–0.4 s.

The effect of pentobarbital is illustrated in Fig. 6. A supplementary dose was quickly injected intravenously. A large but transient fall in P_p was observed, but despite this there was just a slight transient fall in the mean P_{csf} and mean P_p. However, a distinct and longlasting fall in the maximum P_p and maximum P_{csf} occurred due to the profound respiratory depression. This clearly illustrates the importance of pointing out if the mean or maximum values have been used when comparing the P_p and P_{csf}.

The pulse pressure variations were normally 2–3 times larger in the cerebrospinal fluid than in with the perilymph.

Ligation of both external jugular veins did increase the mean P_{csf} and mean P_p by 0.07–0.14 kPa for approximately 1–2 minutes. Ligation on one side, however, did not have any demonstrable effect on the P_{csf} or P_p.

DISCUSSION

Using the described extra-aural approach it was possible to measure the perilymphatic pressure with the ear canal and middle ear intact. This gives excellent possibilities of performing experimental audiophysiological studies as well as investigating the effect of local pressure changes on the ear.

The importance of histological verification of the position of the inner ear cannula was reported by (McCabe & Wolsk (1961) and Weille et al. (1961). Since then this has not been

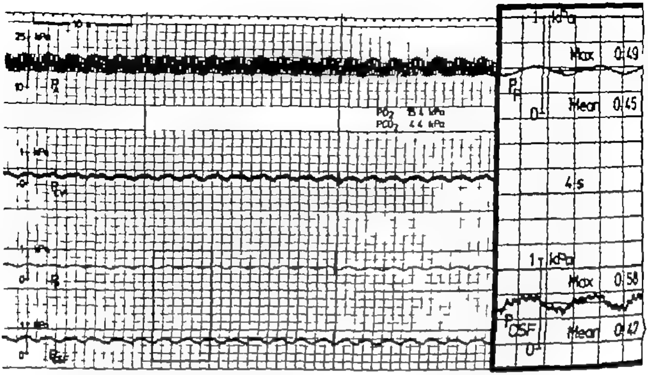


Fig 5 Registration example of the arterial central venous cerebrospinal fluid and perilymphatic pressures The marked area is seen in magnification on the right. This reveals that maximum P_{CSF} during expiration is higher than the maximum P_P while the mean values are approximately equal.

dent s *t* test was used for the maximum P_P and the maximum P_{CSF} levels during a corresponding expiratory phase This showed a significantly ($p<0\ 001$) higher pressure in the cerebrospinal fluid (Table I) The integrated mean value of the P_P and P_{CSF} and calculated during a corresponding respiratory cycle from the registration examples (Fig 5) according to

Table I *A comparison in kPa of the perilymphatic (P_P) and the cerebrospinal fluid pressures (P_{CSF}) using the integrated mean pressure the maximum pressure during expiration and the respiratory induced pressure variations*

Animal no	Mean P	Mean P_{CSF}	Maximum P	Maximum P_{CSF}	Resp. variation P	Resp variation P_{CSF}
11	0.35	0 31	0 39	0.50	0 06	0.27
13	0 45	0 47	0 49	0.58	0 11	0 18
16	1.39	1.35	1 43	1 41	0 07	0.39
19	0.59	0.59	0 68	0 88	0.20	0.54
21	0 75	0 75	0.82	0 88	0.14	0.28
22	1 67	1 70	1 70	1 84	0 07	0.28
24	0.34	0 33	0.39	0 39	0 12	0 15
25	0.38	0.37	0 48	0.54	0.25	0 48
26	1.31	1 43	1 44	1 77	0.25	0.54
27	1 77	1 84	1.84	1 98	0 16	0 27
28	1 16	1 13	1.21	1.29	0.07	0.33
29	0 98	1 01	1.05	1 18	0 08	0.38
30	0 65	0 69	0.85	1 06	0.37	0 74
$m(P - P_{CSF})$	0 014		0 119		0.222	
S D	0 046		0 093		0 107	
t	1 10		4 62		7 47	
P	>0.05		<0 001		<0 001	

tions to study the inner ear hydrodynamics with the present method

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ZUSAMMENFASSUNG

An narkotisierten Katzen sind kontinuierliche Druckmessungen der Perilymphe (P_p), der Zerebrospinalflüssigkeit (P_{csf}) sowie des zentralvenösen (P_{cv}) und des arteriellen (P_a) Blutes durchgeführt worden. Der perilymphatische Raum wurde außerhalb des Ohres zugänglich gemacht, so daß der Gehörgang und das Mittelohr unbeschädigt blieben. Ein konischer Kanal wurde bis hinunter zum Vestibulum im *os temporale* gebohrt. Lage und Verlauf des Kanals wurden später histologisch abgesucht. Eine dünne Metallkanüle mit Gewinde wurde in den Knochen geschraubt. Für die Druckmessungen kamen Kleinstwandler vom Typ Millar zur Verwendung, die eine Beschränkung der meßbedingten Volumenschwankungen auf ein Mindestmaß gewährleisteten. Es ergaben sich gleich große Mittelwerte für P_p und P_{csf}, während der Druck bei maximaler Expiration im Liquor signifikant höher als in der Perilymphe war. Die erfolgte Narkose hatte keinen Einfluß auf die Mittelwerte von P_p und P_{csf}. Die Methode erwies sich als sehr zuverlässig und ermöglicht gehörphysiologische Messungen, ohne Eingriff am Transmissionssystem des Mittelohrs zu erfordern.

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done in the major works on the inner ear pressure

The characteristic feature of almost all previous works on inner ear pressure has been an approach through the opened tympanic cavity or bulla. However this inevitably changes the transmission characteristics of the middle ear (Guinan & Peake 1967)

Investigation of fast pressure changes in small fluid compartments requires a method with minimal volume displacement By using state-of the art microtip transducers and short rigid cannulas it was possible to perform continuous measurements with a volume displacement of 1/1 000 of that of Beentjes (1970) Only one method described earlier (Angelborg & Ågerup 1975) has had less volume displacement but since it only permitted continuous measurement for approximately 1 hour at a time it was not suitable for the present purpose

Leakage of perilymph at the measurement point was a problem in previous methods In the present work this problem was solved by a threaded metallic cannula that was screwed into the bone There has been no sign of leakage even when high pressures were applied to the ear (Densert et al 1978)

This described extra-aural approach made it possible to use an inner ear cannula with a diameter approximately 10 times larger than in other investigations (McCabe & Wolsk 1961 Weille 1961 Martinez 1968 Beentjes 1970 Angelborg & Ågerup 1975) Thereby we have not encountered problems with congestion of the cannula. In earlier works when larger volume displacements were present the diameter of the cannula had a significant damping effect on the measuring system (Beentjes 1970) In the present method the volume displacement is minimal and the diameter of the measuring cannulas are approximately equal The observed time lag and smaller pulse pressure and respiratory variations in the perilymph compared with the cerebrospinal fluid are thus likely to reflect a true difference between the two compartments

According to our results there is no pressure difference between the mean P_P and mean P_{CSF} Earlier authors have reported that the P_{CSF} exceeds the P_P by approximately 0 14 kPa (Martinez, 1968 Kerth & Allen 1963 Beentjes 1970) However these results do not seem to represent the mean values and should therefore be compared with our results of the maximum P_{CSF} and P_P levels during expiration with which they are in agreement Thus the smaller pulse pressure and respiratory induced pressure variations in the perilymph depress the maximum P_P compared to the maximum P_{CSF} To avoid such ambiguities it should be stated whether the mean or maximum values have been calculated when discussing any effect on the P_P and P_{CSF}.

Martinez (1968) reported that ligation of the internal jugular veins caused a sustained increase in the P_{CSF} by 0 27 kPa. Since the present method included cannulation and ligation of one of the external jugular veins it was important to determine that this did not affect the P_P.

Pentobarbital used in our experiments is the most widely used anesthetic agent in studies on inner ear pressure (Weille et al 1958 1961 Kerth & Allen 1963 Martinez 1968 Beentjes 1970 Parker 1977) Barbiturates are known to exert a negative inotropic effect on the heart (Goodman & Gilman 1971) and this may explain the fall in P_A found in our study (Fig 6) Barbiturates can also reduce the cerebral blood flow (Smith & Wollman 1972) and have been used for decompression of intracranial hypertension (Shapiro et al 1975) A similar effect might be expected on the P_P and P_{CSF} because of the close anatomic and hydrodynamic relationships between the two compartments (Lindsay et al 1952 Beentjes 1970) but this has not previously been reported It was therefore important to show that pentobarbital in the dose given had no major influence on the mean P_P.

By using this method continuous reliable recordings of the perilymphatic pressure were made with an intact middle ear transmission system This is the first in a series of investiga

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THE EFFECTS OF INHIBITION OF THE STRIAL Na K ACTIVATED ATPase BY PERILYMPHATIC OUABAIN IN THE GUINEA PIG

S. K. Bosher

From the Ferens Institute of Otolaryngology, The Middlesex Hospital Medical School, University of London, London, England

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Abstract. The endolymphatic effects of perilymphatic ouabain ($2 \cdot 10^{-4}$ M) were followed in 3 guinea pigs using ion-sensitive micro-electrodes, enabling N -related permeability increase to be identified. Investigation of the strial ultrastructural changes in 11 more animals revealed early swelling of the marginal cells, while the intermediate and basal cells became shrunken with characteristically dark-staining cytoplasm. The subsequent cellular alterations were complex. The findings suggest that major function of the Na K -activated ATPase is preservation of the normal intracellular environment, inhibition resulting in widespread indirect effects. General measures of strial function, consequently, do not document just ATPase inhibition.

Although the stria vascularis is considered to have a major role in maintaining the unusual composition of the mammalian endolymph, little is known about the nature of the mechanisms concerned. Early qualitative biochemical and histochemical studies revealed the presence of a membrane-bound Na K activated ATPase in seemingly large amounts (see Schätzle, 1971, for references). But our first significant advance in this respect was the quantitative demonstration by Kuijpers & Bonting (1969) of strial levels of the enzyme equivalent to the high concentrations discovered in electrolyte-transporting tissues and the confirmation of this finding by Matschinsky & Thalmann (1970).

Perilymphatic perfusion with ouabain, a specific inhibitor of Na K activated ATPase, produced a decline in the endocochlear potential to anoxic values, a rise in the endolymphatic Na concentration and ^{22}Na entry rate, and a fall in the K concentration and ^{42}K entry rate (Konishi et al. 1978; Konishi & Mendelsohn, 1970; Kuijpers & Bonting, 1970; Sellick & Johnstone, 1974; Simon et al. 1973). Endolymphatic ouabain, which should be ineffective theoretically since this is the K efflux side, can also cause a rapid decrease in the potential (Tanaka & Brown, 1970). Such experiments, therefore, implied that the ATPase might be directly responsible for some aspects, at least, of the endolymphatic constitution, a view strengthened by the report of a rapid increase in its concentration when the marked elevation of the endocochlear potential occurred during development (Kuijpers, 1974).

However, the concentration of ethacrynic acid resulting in complete abolition of the endocochlear potential and associated with changes in the endolymphatic cation concentrations had practically no effect upon the strial Na K activated ATPase (Kuijpers & Wilberts, 1976; Paloheimo & Thalmann, 1977). Clearly, the endolymphatic alterations could not be due to direct inhibition of the ATPase in this case and its immediate involvement in endolymphatic homeostasis was consequently very doubtful. Recently, this conclusion was confirmed when the normal ratio of strial active K to Na transport was shown to be substantially higher than any known ATPase coupling ratio (Bosher, 1980*a*).

Nevertheless, inhibition of the Na K activated ATPase by ouabain greatly diminishes energy utilisation by the stria vascularis (Kusakari et al., 1978) and reduces its respiratory rate by about half (Marcus et al.

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B Carlborg M.D
Department of Otolaryngology
Malmö General Hospital
University of Lund
Malmö
Sweden

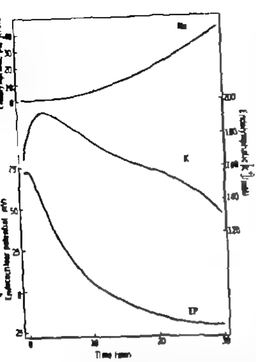


Fig. 1. The changes in the endocochlear potential (14 experiments) and the endolymphatic cation concentrations (3 experiments) during scala vestibuli perfusion with 10 M ouabain showing the means and S.E.M. (*stippled area*). The K findings are probably influenced by interaction between the ouabain and the K -sensitive microelectrodes

by the scala vestibuli openings from its usual mean level of 81.3 mV to 71 8±1 8 mV (n= 14) a reduction comparable to the 13 6 mV previously observed in the rat (Bosher 1979) The initial effect of the ouabain perfusion varied In 9 animals there was an immediate rise of 1.±0.3 mV before the potential began to decline at 30 sec in 4 there was an immediate fall in 1 there was no change for 45 sec when the potential started to decrease Apart from the latter experiment, no measurable latent period occurred the potential beginning to alter as soon as the perfusion was initiated

The reason for the transient increase is not obvious. In order to minimise the possibility of pressure effects the perfusion rate was slow the scala vestibuli openings were generous and no attempt was made to obtain a tight fit of the perfusion pipette No change was observed in control experiments and any possible basilar membrane displacement would tend to cause a decrease in the potential

A difference in K concentration between the perfusate and the perilymph might also affect the potential but such an action has only been seen with scala tympani perfusion This was one reason for selecting scala vestibuli perfusion the other being that it avoids all danger of interference with the metabolic supply of the organ of Corti It is difficult, consequently to attribute the potential increase to some technical artefact. Hence it might represent the result of temporary stimulation of the Na K -activated ATPase by the ouabain before its main inhibitory effect a sequence present in vitro (Kuijpers & Bonting 1969)

Once it had commenced the potential decrease was moderately rapid at first (Fig. 1) the polarity changing at 12.5±0.5 min (n=8) Later the rate of fall declined and a minimum level of −20 7±1 9 mV was not reached until 27 7±1.3 min (n=5) which compares with 9 min in anoxia. Subsequent i v ethacrynic acid (40 mg kg⁻¹) and anoxia had no effect, confirming that the ouabain had completely abolished the positive component of the potential.

(b) *Endolymphatic Na concentration* The endolymphatic Na concentration began to increase from its control level of 0 64±0 14 mM (n=3) immediately the ouabain perfusion was started Initially the rate of change was very slow and the concentration at 5 min had only risen to 1 77±0 11 mM (n=3) but the rate subsequently increased so that the concentration at 30 min was 43 0±5 6 mM (n=3) (Fig 1)

These concentration alterations are impossible to interpret by themselves because of the concomitant decrease in the endocochlear potential They were normalised therefore with respect to the measured *electrochemical* gradient and the rather complicated result is shown in Fig. 2. The first portion of the curve represents the early progressive diminution in the activity of the Na -transporting processes

1978) Serious problems remain accordingly about the true function of the enzyme about the way its inhibition brings about the strial and endolymphatic abnormalities found and about the reason for its high concentration

Surprisingly no accounts of the electron microscopic features of ouabain inhibition are available except for the description by Simon et al (1973) of obliteration of the normal wide intercellular spaces in the dark-cell epithelium of the frog labyrinth which were thus considered to be involved in active ion transport These authors published no electron micrographs of their ouabain treated material and it is noteworthy that similar spaces are not visible in the mammalian stria vascularis For these reasons it was thought worthwhile to investigate the ultrastructural effects of ouabain upon the stria whose functional activity was monitored by measurement of the endolymphatic derangements in an attempt to provide more information about the role of the Na^+ K^+ activated ATPase

METHODS

A full description of the experimental techniques and procedures has been published in a previous paper (Bosher 1979) In summary the animals used were female Caesarian originated barrier sustained Hartley-derived albino guinea pigs (Charles River) 200–300 g in weight whose ears had not been subjected to the effects of either infection or antibiotics Surgical anaesthesia was maintained by neuroleptic agents without the administration of muscle relaxants and the animals temperature was carefully controlled at 37°C (±0 4°C)

A small fenestra was made over the stria vascularis of the left basal turn for the insertion of micro-electrodes into the scala media Then two openings were made into the scala vestibuli a quarter turn on either side of the scala media fenestra Ouabain was perfused as a 2×10^{-3} M solution in artificial perilymph through the apical opening at a rate of 3.5 μl min⁻¹

Three initial experiments were performed in which the endolymphatic Na and K concentrations were followed by means of ion-sensitive micro-electrodes in addition to the endocochlear potential These experiments were terminated with anoxia produced by the i injection of 2 ml air In each case the terminal procedure confirmed that 100% inhibition of the positive potential-producing and cation transporting processes had occurred at the estimated time

In the 11 principal experiments the endocochlear potential only was recorded with a conventional micro-electrode The ouabain perfusion was terminated by fixation for histological examination at predetermined points (2 animals at each point the perfusion durations being as indicated) namely immediately after onset (1 17 min 1 75 min) and after the endocochlear potential had decreased by 25% (3.5 min 3 5 min) 50% (5 min 7 min) 75% (12 min 13 min) and 100% (31 min 36 min) In addition fixation was carried out in one animal after 23 min perfusion (97% potential inhibition) because of the gap between the 75% and 100% times

Fixation was achieved by irrigation with phosphate-buffered (pH 7 2–7 4) 1% osmium tetroxide at 4 C through the scala vestibuli fenestrae the round and the oval windows The animal was decapitated the left cochlea removed and immersed in the same fixative for another 2 hr Specimens were embedded in Spurr s resin thin sections of the ouabain perfused region were prepared using a Reichert OM3 ultramicrotome and examined in a Philips EM 300 electron microscope after staining with uranyl acetate and lead citrate

The results are routinely given in the form mean standard error of mean (number of experiments) The Na and K findings are expressed as concentrations (not activities)

RESULTS

1 *Functional effects*

(a) *Endocochlear potential* The normal potential during the control period was reduced

processes was much more marked and accompanied by a similar effect upon the cell bodies while greatly increased electron-density of the intermediate cell cytoplasm constituted a new and distinctive pathological feature (Fig. 3) Although relatively normal intermediate cells could occasionally be found (Fig. 3C) the usual picture was of dark contracted cell bodies and processes associated with enlarged intercellular spaces (Fig. 3A)

All the marginal cells continued to look completely normal However it became evident at this stage that the basal cells were also involved in exactly the same way as the intermediate cells. Their processes were severely shrunken and although their bodies were less affected small intercellular spaces were visible In addition their cytoplasm was uniformly electron-dense making the extensive arborisations of the processes particularly noticeable (Figs 3A B) In both intermediate and basal cells the intracellular organelles were somewhat obscured but did not seem abnormal

(b) *50% inhibition* In one animal (5 min perfusion) slight to moderate swelling had occurred in about 20% of the marginal cells dispersed singly or in small groups. In the other (7 min perfusion) the incidence of swollen cells had increased to approximately 80% roughly half being severely affected Initially the cell surface developed localised protuberances (Fig. 4A) followed by generalised swelling and protrusion of the superficial portion of the cell into the endolymph (Fig. 4B) The sub-plasmalemmal region was maximally involved and was often clearly distinguishable from the deeper cytoplasm and organelles

A second major development was the appearance in the marginal cells of many severely-damaged mitochondria with large translucent spaces (Figs. 3D 4B) Such mitochondria were present in all parts of the cell and although more common in severely swollen cells were also a feature of relatively normal-sized ones No mitochondrial alterations were visible in the intermediate and basal cells These cells were unchanged in one animal (Fig 3D) but were less electron-dense in the other It was noticeable that intercellular spaces were only found where the marginal cells were not swollen Elsewhere the spaces were obliterated by the enlarged processes (Fig. 3D)

(c) *75% inhibition* Surprisingly the marginal cell swelling was decreased overall and had not increased as expected In both animals about 20% of the cells were still considerably enlarged but most of the remainder were only slightly swollen and, in one animal approximately 20% were normal in shape The surface plications invariably present in the worse-affected cells (Fig 4D) suggested regression of a previously more-advanced state as did the membrane irregularities found in the others (Fig 4C) Whether all or merely a proportion of the cell population became greatly enlarged at some stage or other during the pathological process cannot be determined from the histological evidence alone The mitochondria were again grossly abnormal although the other organelles seemed uninvolved (Fig 4C D)

The intermediate and basal cells were universally less electron-dense than before (Fig 4C) and often appeared to have returned to their usual size and staining character In a few regions the intermediate cell processes were unquestionably swollen while no intercellular spaces were visible even in areas without enlarged marginal cells

(d) *90–100% inhibition* The marginal cell swelling had continued to decrease or had disappeared in the two 100%-inhibited animals When present, it was only relatively slight but the endolymphatic surface of such cells was typically irregular (Fig. 5A) Many cells which were no longer swollen exhibited surface folding, although some appeared quite normal Localised structural derangements were still evident in the majority of mitochondria at high magnification However little abnormal could be seen at low magnification and the only new feature was the appearance of unusually large

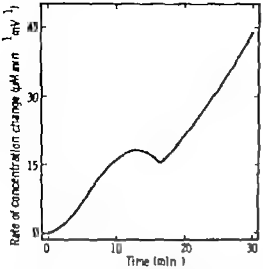


Fig 2 The changes in the endolymphatic Na concentration shown in Fig. 1 normalised with respect to the electro-chemical gradient for Na giving the mean and S E M (*stippled area*) of the 3 experiments

No stable period appears because transport was not completely abolished before 30 min unlike the situation after anoxia (Bosher 1979) However a clear change in the character of the curve is visible at 16 3±1 8 min (n=3) when the previous tendency for the rate to decline is reversed and a period of progressive increase is initiated relatively abruptly The sodium concentration at this time was 13 3±1 9 mM (n=3)

The sudden rise in the normalised rate of flow of Na^+ into the endolymph indicated an increase in the Na^+ permeability of some portion at least of the endolymphatic membranes A similar phenomenon has been found after anoxia in normal animals (Bosher 1979) In the present experiments where it occurred during the ouabain perfusion it was not repeated for a second time during the terminal anoxic period which revealed that the two effects were not additive More precise calculation of the ouabain action upon the active transport mechanisms from the anoxia findings (Bosher 1980*a*) was considered inadvisable because of the concentration-associated permeability changes during the perfusion

(c) *Endolymphatic K concentration* The K^+ concentration appeared to increase rapidly from the control level of 166.2 mM to a peak of 193 3 mM before decreasing to 130 4 mM at 30 min (Fig 1) The initial rise thus seemed to be far greater than the one in the endocochlear potential and it was difficult to attribute the effect solely to early stimulation of the K transporting mechanisms This difficulty in interpretation led to further investigation, which revealed that ouabain strongly interacts in a most complex fashion with the K sensitive micro-electrodes used in the experiments (Bosher 1980*c*)

The apparent alterations in the K^+ concentration might well have been due therefore to an interaction of this nature If this should be the case then perilymphatic ouabain would have to be transported quickly into the endolymph The active transport of ouabain has been reported in other systems (Lauterbach 1975) but further study is clearly essential before any conclusion about the state of affairs in the inner ear is possible especially in view of the consequences of such a situation Meanwhile the present K results can only be taken at the most as indicating an overall qualitative decrease in the endolymphatic K concentration during the ouabain perfusion

2 *Morphological effects*

(a) *0–25% endocochlear potential inhibition* The stria vascularis in the animal examined after 1 17 min perfusion was entirely normal but shrinkage of some of the intermediate cell processes with the consequent formation of enlarged intercellular spaces had commenced in the 1 75 min perfusion animal These changes were scattered in distribution and, in general resembled the initial appearances in ethacrynic acid intoxication (Bosher 1980*b*). They did not occur after control perfusions of up to 30 min

More widespread alterations of the same type were present in one of the 3.5 min perfusion (25% inhibited) animals still interspersed with normal-looking regions In the other the shrinkage of the intermediate cell

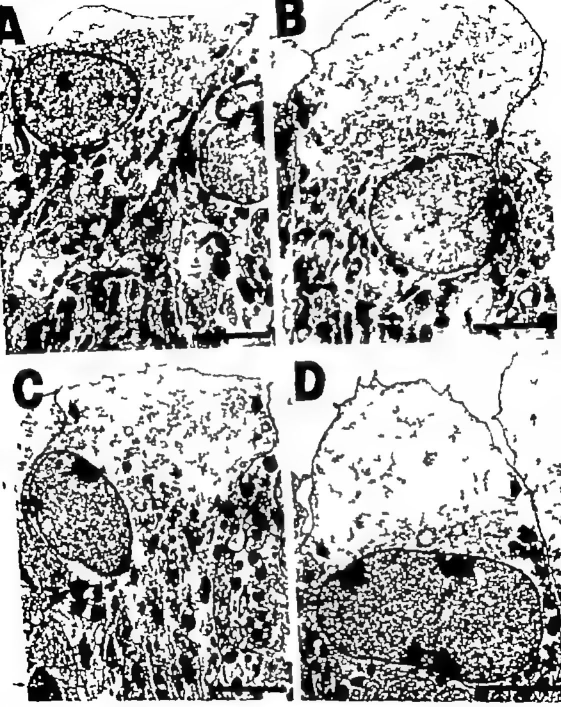
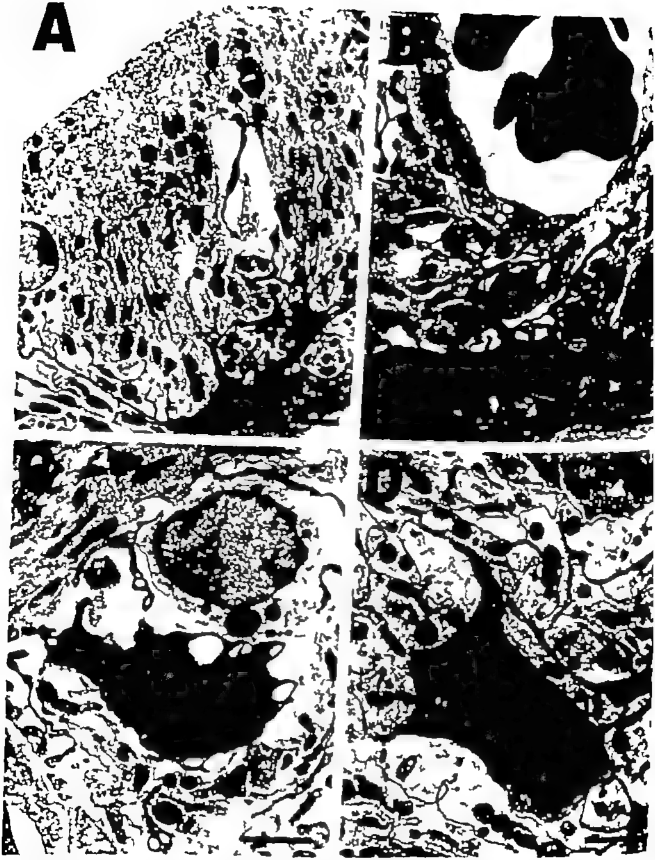


Fig. 4. Marginal cell alterations. (A) 50% inhibition. Early swelling of the sub-plasmalemmal region. (B) 50% inhibition. Severely swollen cell with 'turned' tight junctions (arrow) and marked mitochondrial changes. (C) 75% inhibition. The irregularity of the surface membrane and the [illegible] ns (arrows) are typical. The intermediate cells are less electron-dense than in Fig. 3 (A). (D) 75% inhibition. The position of the tight junctions (arrows) confirms that the maximum degree of swelling (shown here) is less than in B. Characteristic surface plications are present. Bars: 4 μm.



F g 3 (A) ,5 % inhibition. The intermediate and basal cell processes are contracted and very electron-dense making the basal cell arborisations clearly visible (B) 25 % inhibition. Intercellular spaces are present between the dark basal cells (C) 25 % inhibition A les deeply-stained intermediat cell contrasts with more typically affected one (D) 50 % Inhibition The previous intercellular spaces are replaced by swollen marginal cell processes th grossly abnormal mitochondria. Bars μm.

regular vesicles often multiple mainly in cells with signs of past enlargement (Fig. 5 A)

The most striking change was the development of marked and generalised swelling of the intermediate cells (Fig. 5) It was associated with severe damage to the mitochondria and dilatation of the rough endoplasmic reticulum. Many processes looked almost empty due to the extreme dispersion (and possible disappearance) of the organelles By contrast the basal cells and processes remained their usual size

The state of the one animal examined after 23 min perfusion was much closer to the 75%-inhibited than the 100%-inhibited group despite 97% inhibition of the endocochlear potential Marginal cell enlargement comparable to that seen in the 75%-inhibited animals was common and mitochondrial vacuolation was prominent, if reduced in extent The intermediate cells were not definitely swollen although dilatation of the rough endoplasmic reticulum had occurred

DISCUSSION

The functional effects of ouabain described here are in general accord with the earlier findings summarised in the introduction. The only aspect requiring further consideration is the accelerated Na entry probably due to increased membrane permeability which arose when the endolymphatic Na concentration reached an average level of 13.3 mM This closely resembled the situation found in straight forward anoxia, where the mean critical Na concentration of 11.3 mM (Bosher 1979) was not significantly different. Anoxia induced in the ouabain-treated animals, after the Na concentration had passed this critical level and an increase in permeability had already taken place had no additional effect strongly suggesting that the two phenomena have a common basis The nature of the responsible membrane alterations remains obscure but their occurrence in fully-oxygenated animals has now been demonstrated

As regards the morphological effects upon the stria vascularis, which have not been described before all three types of cell are clearly affected in a complex fashion The marginal cells become swollen initially after perilymphatic perfusion for 3.5 to 5 min In other tissues, ouabain often produces no alteration in cell volume but, where it does it always seems to cause an increase (Blom & Helander 1977 Cooke 1978 Macknight & Leaf 1977) so the reaction of the marginal cells follows the general pattern Recently Gupta et al. (1978) have found evidence that the sub-plasmalemmal zone in ileal mucosal cells forms a channel for the preferential movement of the ions being transported by the cells The marginal cell swelling commenced and was always greatest in this region which might indicate the existence of the same kind of situation although further investigation is obviously required

With continued application of the ouabain, the swelling should have persisted and the reason for its subsequent disappearance is puzzling There is no question of any recovery and it must be supposed that some profound abnormality of the cell membrane developed allowing a massive redistribution of osmotically active substances and water between the cells and their surroundings To what extent this is related to the coincident Na -dependent increase in the overall permeability of the endolymphatic system or to the secondary actions discussed later cannot be determined Although the cause of the volume decrease is unknown, it testifies to additional intracellular changes in the marginal cells, as do the mitochondrial derangements These resemble those described in ethacrynic acid intoxication (Brummett et al. 1977) and are almost certainly associated with decreases in energy production and oxygen consumption.

Turning next to the intermediate cells, their early shrinkage resembles that which has been widely reported after ethacrynic acid administration In this sense its occurrence is not unusual but as will be realised from the foregoing discussion the shrinkage does represent

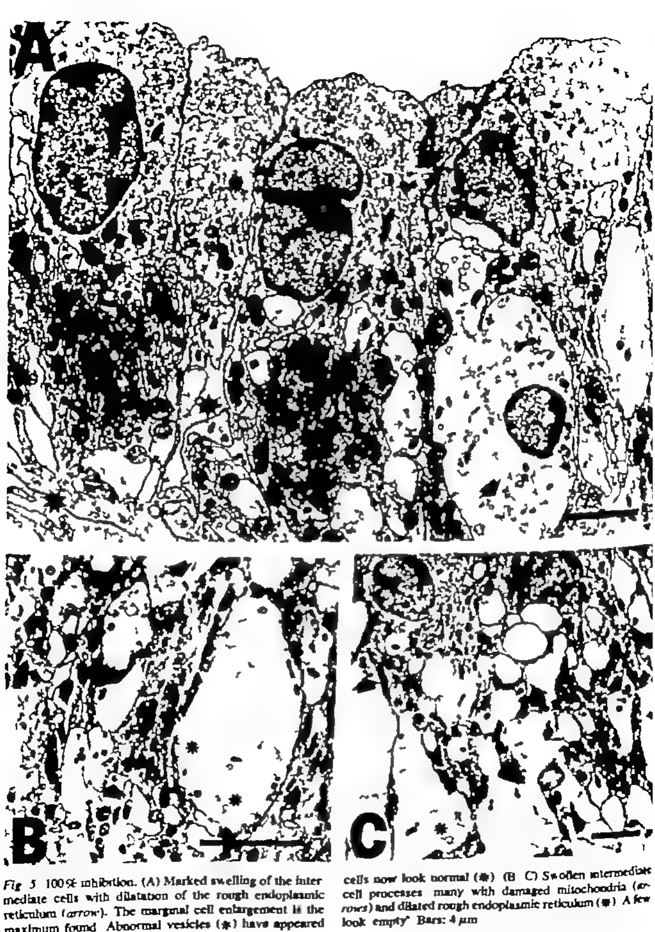


Fig 5 100% inhibition. (A) Marked swelling of the intermediate cells with dilatation of the rough endoplasmic reticulum (*arrow*). The marginal cell enlargement is the maximum found. Abnormal vesicles (*) have appeared but the mitochondrial changes are not obvious. The basal cells now look normal (*). (B, C) Swollen intermediate cell processes, many with damaged mitochondria (*arrows*) and dilated rough endoplasmic reticulum (*). A few look 'empty'. Bars: 4 μm

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S K Bosher
Ferens Institute of Otolaryngology
The Middlesex Hospital Annexe
Cleveland Street
London W1P 5FD
England

an exceptional reaction to ouabain in comparison with other types of cell No exact correlation exists between its degree and the size of the intercellular spaces since these are partly influenced by the marginal cell alterations The origin of the electron-dense staining of the intermediate and basal cell cytoplasm cannot be identified but it seems to indicate some change in the intracellular state At any rate the dark appearance is not the sole result of cell shrinkage for it does not occur after ethacrynic acid even if it does disappear with the later increase in cell volume

Finally there remains the question of the normal function of the Na^+–K^+ activated ATPase Here the most informative findings were the volume changes produced in both the marginal and intermediate cells within minutes of the application of ouabain by perilymphatic perfusion Elsewhere volume changes are accompanied by progressive and ultimately severe alterations in the intracellular ionic composition including Ca^{2+} (e g Macknight & Leaf 1977) and it thus seems reasonable to conclude that similar alterations occur very quickly in the strial cells This is relevant because the ionic composition has been shown to affect a wide variety of cell activities ranging from ribosomal synthesis to transepithelial transport (e g Civan 1978) and may even partly control the permeability of the tight junctions (Reuss et al 1979) In the stria the subsequent morphological developments also point to considerable abnormalities of cell organisation Ouabain therefore almost certainly causes widespread indirect effects in addition to its specific primary inhibition of the ATPase

This conclusion has at least three consequences Firstly any measure of strial function (e g endolymphatic composition strial energy utilisation strial oxygen consumption) will be influenced by many (and possibly all) of these indirect effects so that such measures will not provide an accurate assessment of the results of simple ATPase inhibition alone Secondly strial ATPase inhibition will be rapidly followed by undesirable intracellular changes and one of the major actions of the enzyme seems to be the preservation of the normal internal environment of the strial cells This would be important in an actively-transporting tissue and may well be its major role Thirdly any toxic agent or pathological process which produces similar intracellular ionic alterations will have a similar diversity of indirect effects

ACKNOWLEDGEMENT

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ZUSAMMENFASSUNG

Die Auswirkung von perilymphatisch verabreichtem Ouabain (2×10^{-4} M) auf die Endolymphe wurde bei 3 Meerschweinchen mit Hilfe einer ionensensitiven Mikroelektrode die die Na -abhängige Permeabilitäts-Veränderung erfahren kann verfolgt. Die ultrastrukturelle Untersuchung der Stria bei 11 weiteren Meerschweinchen zeigt eine frühzeitige Schwellung der marginalen Zellen, aber eine Schrumpfung der intermediären und basalen Zellen mit einem charakteristisch dunkel gefärbten Cytoplasma. Die späteren Zellveränderungen waren komplex Die Befunde weisen darauf hin, daß eine Hauptfunktion der Na –K -aktivierten ATP-ase die Erhaltung der normalen intracellulären Milieus ist. Eine Hemmung hat ausgedehnte indirekte Effekte zur Folge Messung der allgemeinen Stria-Funktion zeigt demnach nicht nur ATP-ase Hemmung.

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ble 1 *A summary of the velocity patterns of different eye movements in all the patients*
e type and site of lesion are described. Fast = fast phase velocity Slow = slow phase velocity DP = directional
epoonderance. CP = canal paresis. N = normal. ↓ = decreased

t.	Age	Type of lesion	Side	Level	Pursuit velocity	Saccade velocity	Rotation fast	Rotation slow	OKN fast	OKN slow	Optovest fast	Optovest slow	Caloric slow
A	65	Vascular	Right	Lateromed.	↓	↓	↓	N	N	N	N	N	DP right
A	17	Tumour	Bilateral	Pontine	↓	↓	↓	↓	↓	↓			CP left
H	71	PSP	Bilateral	Supranuclear	↓	↓	↓	N	N	N	↓	↓	CP left
J	66	Vascular	Bilateral	Medullar?	↓	■	■	N	N	N	■	↓	CP right
N	71	Vascular	Right	Medullar	↓	↓	N	N	N	N	N	N	CP left
P	73	Vascular	Left	Pontine	↓	↓	N	↓	N	↓	↓	↓	DP right
R	71	PSP	Bilateral	Supranuclear	↓	↓	N	↓	N	↓	↓	↓	CP right + DP right
R	58	Vascular	Right	Pontine	↓	↓	↓	N	N	↓	N	↓	CP left
S	72	Vascular	Left	Lateromed	↓	↓	↓	N	N	↓	↓	↓	
W	46	Vascular	Right	Pontine	↓	↓	↓	N	N	↓	↓	↓	DP right

as possible at the light diodes At least five saccades were recorded for each angle and direction

Caloric nystagmus was produced by a routine caloric test with warm and cold water and the test was conducted in darkness (Henriksson et al 1972)

Nystagmus caused by *rotation* was produced in a chair that was accelerated with 2 1 rad s^{-2} within one second to a constant velocity of 2 1 rad s After one minute of rotation the chair was decelerated The nystagmus in the per and postrotatory *responses* were measured The test was performed in darkness with both eyes open

To elicit *optokinetic* nystagmus an optokinetic cylinder covering the whole visual field was rotated around the subject at a constant velocity of 1.6 rad s

Optovestibular nystagmus was achieved with a standardized visual surrounding and the patient was again rotated as *in the rotation* test, but now with eyes opened

An electro-oculographic technique was used Horizontal eye movements were recorded binocularly vertical eye movements monocularly from both eyes The recorder used was an ink-jet ENG recorder (Mingograph M81) with an upper cutt-off frequency of 15 Hz. The paper was fed at a speed of 100 mm s The calibrations at 0 35 and 1 05 rad were done twice at the beginning and in the middle of the test.

In the present study the *main interest* was directed towards the peak velocities in the movements of the eye *Normal* values for the peak velocities in fast eye movements (Henriksson et al 1980) and smooth pursuit eye movements (Schalén 1980) and described elsewhere. A velocity outside 95% confidence limit from the normal mean value was regarded as pathological Thus in the smooth pursuits the maximal velocity was estimated during the time the patient was following the target (i.e without superimposed saccades) (Schalén 1980) In the voluntary saccades and the *fast* phases of nystagmus the peak velocities were measured and arranged in six different groups according to the amplitudes (Henriksson et al 1980) *In the slow* phases of nystagmus the peak velocity was estimated at the culmination response

RESULTS

The most characteristic finding was that all patients had impaired ability to perform smooth pursuits. The second most characteristic finding was that 9 out of 10 patients had significantly decreased velocities in voluntary

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EYE MOVEMENTS IN BRAINSTEM LESIONS

C Wennmo and B Hindfelt

From the Departments of Otorhinolaryngology and Neurology University Hospital Lund Sweden

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Abstract The eye movements of 10 patients with brainstem lesions were examined using an electro-oculographic technique An analysis was made of the responses in the following tests smooth pursuit saccade rotation optokinetic optovestibular and caloric. Chief interest was directed towards the peak velocity The results were compared with the corresponding velocities in 20 normal subjects In the same patient, one or several types of eye movements could be normal while others were pathological In the whole group voluntary eye movements (smooth pursuits and voluntary saccades) were more vulnerable than reflexive ones (nystagmus). Next to the smooth pursuit test and the voluntary saccade test the optovestibular test was the most sensitive in discriminating patients with brainstem lesions from normal subjects

Numerous neurons in and between the vestibular nuclei the paramedian pontine reticular formation (PPRF) and the eye motor nuclei are involved in the control of eye movements (Raphan & Cohen 1978)

The PPRF is considered to be the major target for most of these neurons (Bender & Shanzer 1964 Cohen & Feldman 1968 Cohen et al 1968) In the PPRF *phasic* units active during fast eye movements are located in the rostral part and *tonic* units active during slow eye movements are located more caudally or around the abducens nucleus (Luschei & Fuchs 1972 Keller 1974 Henn & Cohen 1975) The same phasic units discharge before any fast eye movement either visually or vestibularly evoked (Keller 1974) During fixation all saccades are inhibited by *pausing* units scattered throughout the PPRF (Keller 1974)

The aim of this study was to find out how various types of eye movement were disturbed in patients with brainstem lesions It also was our intention to map out how various distur bances in the velocities of eye movements were combined

MATERIAL AND METHODS

The material consisted of 10 consecutive patients with brainstem lesions who were thoroughly examined by a neurologist The diagnosis was based on case history and neurological findings Whenever possible the lesion was topographically defined The work-up included computerized tomography EEG neuro-ophthalmological and neuro-otological examinations Seven patients had brainstem infarctions at various sites 2 had progressive supranuclear palsy and one had a tumour in the fourth ventricle

Twenty healthy subjects whose ages ranged from 23 to 72 (mean age 48) years were used as controls

Smooth pursuit eye movements were induced by projecting a light spot of 30 mm diameter covering an angle of 1 05 rad on a screen The spot moved at constant velocities of 0 17 0 35 0 52 0 70 0 87 and 1 05 rad s⁻¹ in front of the patient and a new spot was substituted when the other one disappeared At least five recordings were made at each velocity and direction (Schalén 1980)

Voluntary saccades were induced by asking the patient to look at light diodes placed horizontally in front of the subject at angles of 0 08 0 17 0.35 and 0.52 rad on both sides of the midline (Henriksson et al 1980) the patient being told to look alternatingly as fast

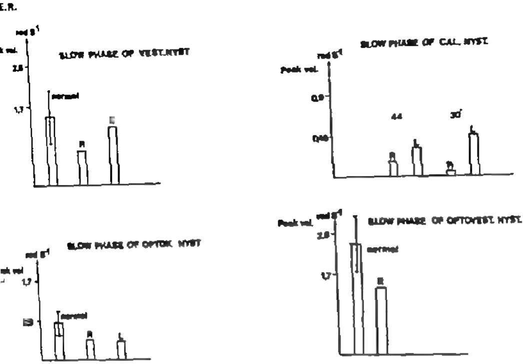

Fig. 2. Velocity patterns of slow components in different types of nystagmus in the same patient as in Fig. 1. R = right, L = left.

preserved while the saccadic mechanism was disturbed indicating a dissociation between saccades and fast phases of nystagmus.

The slow phases in nystagmus in the same patient were affected in various ways (Fig. 2). The peak velocity of the slow phase of caloric nystagmus, of angular acceleration induced nystagmus and of optovestibular nystagmus was always reduced when directed towards the right, while the optokinetic responses were reduced in both directions. Thus a dissociation between vestibular and optokinetic slow phases had taken place.

Another patient, a male with a tumour in the fourth ventricle (B. A.) had a markedly reduced saccadic velocity (Fig. 3). For example with 0.7 rad amplitude the velocity was only 2 rad s (normally 7.5 rad s). During smooth pursuits his eyes lagged behind the target at target velocities between 0.17–0.6 rad s. However, at higher target velocities the smooth pursuits were within normal range and could not then be distinguished from the saccadic return eye movements (Fig. 4) because of almost identical shape and velocity. The dissociations in this patient are reflected by the presence of voluntary eye movements while the nystagmus was completely absent.

A third patient (E. J.) had an infarction in the brainstem (Fig. 5). She had normal velocities in both components in the rotatory test as well as in voluntary saccades. However the ability to follow voluntarily was diminished indicating a dissociation between mechanisms regulating the two types of voluntary eye movement (voluntary saccades and smooth pursuits).

DISCUSSION

The reason for selecting the peak velocity as the best parameter in discriminating between

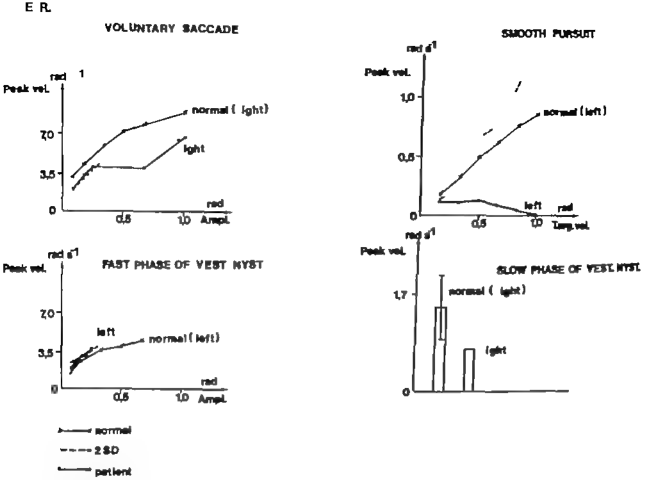

Fig 1 Velocity patterns in voluntary saccades smooth pursuits and fast and slow phases in angular acceleration induced nystagmus in a patient with PSP Only eye movements to one side are depicted (the most pathological ones) Dashed lines indicate 2 S.D on each side of the mean peak values in normals.

saccades The results are summarized in Table I

In the rotation test the peak velocity in the fast phase was significantly reduced in 6 out of 10 patients the figures for optokinetic test and optovestibular test being one out of 10 and 5 out of 9 respectively

The most common abnormality in the slow phases was a decreased slow phase velocity in the optovestibular test (7 out of 9 patients) In the rotatory test 3 out of 10 were impaired and in the optokinetic test 6 out of 10 Three patients had significantly decreased velocities in both the rotation and the optokinetic test.

Sometimes in the same individual the velocity in one type of nystagmus could be normal while the velocity in the same component in other types of nystagmus was decreased (Table I)

In caloric nystagmus the patients showed either directional preponderance (DP) or canal paresis (CP) and none had normal caloric responses Four subjects had a DP and 6 had a CP Four of the patients had a spontaneous nystagmus

The findings in some of the patients are accounted for in detail in order to map out how various components of eye movements in the same individual are differently disturbed

One patient (E R) had a progressive supranuclear palsy (Fig 1) The pathological findings in this patient were decreased velocity in voluntary saccades as well as in smooth pursuits and slow phases of nystagmus in rotation test and optokinetic test while the velocities of the fast phases in the same nystagmus were normal In this patient the eye motor mechanism producing fast

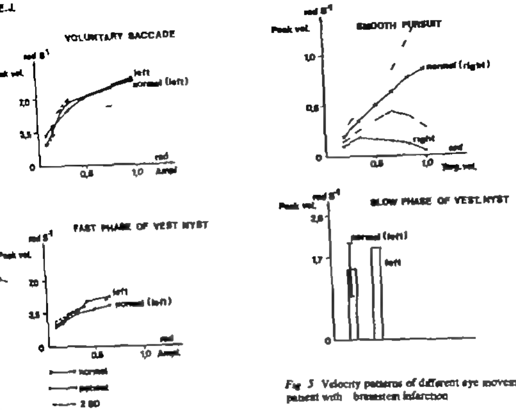

Fig. 5. Velocity patterns of different eye movements in patient with brainstem infarction.

The velocity of the two types of voluntary eye movement was the most sensitive indicator of a pathological process in the pons. This is consistent with findings by other investigators (Baloh et al. 1975, 1977). The smooth pursuits, however, are disturbed in the same way in other neurological disorders, such as cerebellar lesions (Baloh et al. 1977) and cortical lesions (Hoyt & Daroff 1971, Sharpe et al. 1979).

In the same way the voluntary saccades may be more sensitive than reflexive eye movements because of longer pathways and presumably a larger number of synapses. Certainly we also have to consider the difference in the interaction between the agonist and the antagonist in the different types of eye movements (Shimazu 1972). According to previous experimental findings in animals (Keller 1974) the same units in PPRF are active during all fast eye movements, whether voluntary saccades or fast phases of nystagmus. Thus it could be expected that in a brainstem lesion all fast eye movements would be equally affected. As the voluntary saccades in this study were more seriously disturbed than the fast phases of nystagmus, this model of eye motor function cannot explain the present findings. A difference in the neuronal organization of voluntary saccades and fast phases of nystagmus is supported by previous findings in humans (Henriksson et al. 1980), where it was concluded that the peak velocity in voluntary saccades were faster than the peak velocity in the fast phases of nystagmus.

Just as the peak velocity in voluntary saccades and fast phases of vestibular nystagmus could be unequally affected, the peak velocity in the fast phases of optokinetic nystagmus or optovestibular nystagmus could be normal

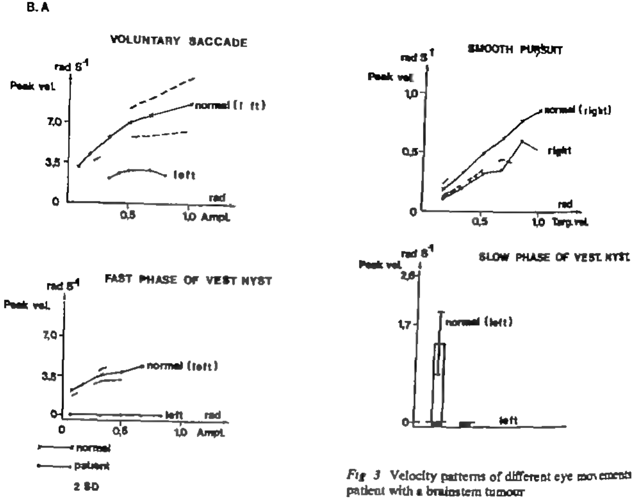

Fig 3 Velocity patterns of different eye movements in a patient with a brainstem tumour

healthy and diseased subjects may need some explanation In the smooth pursuits the ability to hold the object constantly in the fovea is reflected by the maximal velocity (Robinson 1965) A normal peak velocity in the saccades and fast phases of nystagmus indicates intact synchronous firing of the neurons which trigger the right amplitude (Keller 1974) The peak velocity in the slow phase of nystagmus measures the maximal gain in the vestibulo-ocular reflex arch (Melvill Jones & Milsum 1971) Furthermore the velocity parameter seems to be coded in pontine neurons (Keller 1974)

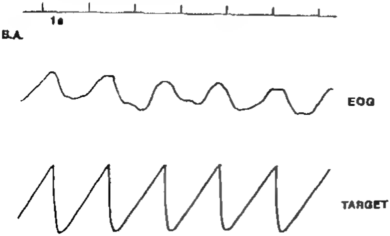

Fig 4 EOG recordings f pursuit eye movements f

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THE POST NATAL MAMMALIAN LABYRINTHINE SECRETORY EPITHELIUM IN VITRO

Matti Anniko

From the Department of Oto-rhino-laryngology, Karolinska sjukhuset and King Gustaf V Research Institute, Karolinska Institutet, Stockholm, Sweden

(Received August 7, 1979)

Abstract. Newborn and mature inner ears from the guinea pig and the CBA/CBA mouse were explanted to an in vitro system. Special attention was paid to the secretory regions of the labyrinth showing surviving cells during the investigated period of 1 week on organ culture. Cells comprising the stria vascularis and the dark cells of the vestibular organs were both morphologically rather well preserved. Degenerative cytological alterations became apparent towards the end of the culture period.

The complexity of the structure and function of both the newborn and the mature mammalian inner ear has made their in vitro preservation extremely difficult with regard to an intact ultrastructural morphology. In analysing the organ culture technique, Anniko (1979) described how in both the CBA/CBA mouse and in the guinea pig hair cell characteristics rapidly were altered after only a few days in vitro with a loss of specific hair cell characteristics, amongst others showing sensory hair fusion.

As opposed to this, during the embryologic development of the CBA/CBA mouse a rather mature configuration of the general shape of the organs and individual hair cells can be obtained in the in vitro environment as regards the vestibular part of the inner ear (Anniko et al. 1979). The innervation of the vestibular hair cells with fully developed nerve chalices and efferent nerve endings is, however, not completed until the first weeks post partum (Nordemar & Anniko, 1979, to be published). The maturation of the cochlea occurs mainly after birth although its organogenesis is usually completed at the time of partus (Sher, 1971; Anniko & Wersäll, unpublished observations). When organ cultures pass the period of time corresponding to partus, an instability of the in vitro system gradually occurs with a subsequent degeneration of the inner ear specific morphology within approximately 1 week or somewhat more.

Studies on the post natal inner ear secretory epithelium in vitro are lacking in the literature. The present study is an analysis of cells comprising the secretory epithelium in both the vestibular and cochlear parts of the post natal labyrinth in the CBA/CBA mouse and the guinea pig.

MATERIAL AND METHODS

Material

Fourteen inner ears (7 animals) from both newborn and mature guinea pigs and CBA/CBA mice, respectively, were explanted to the in vitro system. Thus the whole material consisted of 14 guinea pigs and 14 CBA/CBA mice.

Methods

The membranous labyrinth was dissected free from surrounding bone or cartilage to at least 3/4 of its extent. The cochlear and the vestibular organs were separated from each other but preserving as much soft tissue as possible

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while the peak velocities in the corresponding phases of other types of nystagmus could be significantly decreased

Similar differentially disturbed peak velocities were found in the slow phases of various types of nystagmus

The velocity patterns of different eye movements described in the present study indicate a neuronal organization at the pontine level possibly in the PPRF, different for all eye movements The findings might also help to interpret and understand how normal eye movements are brought about

ZUSAMMENFASSUNG

Die Augenbewegungen von zehn Patienten mit Hirnstammläsionen wurden mittels Elektro-okulografischer Technik untersucht. Eine Analyse der maximalen Geschwindigkeit in Blickfolge Sackaden per und post rotatorischer Nystagmus optokinetischer Nystagmus optovestibularer Nystagmus und kalorischer Nystagmus wurde ausgeführt Die Resultate wurden mit der maximalen Geschwindigkeit der Augenbewegungen bei zwanzig Normalpersonen verglichen In der Gruppe der Patienten mit Hirnstammsläsionen fielen ein oder auch mehrere Augenbewegungsteste bei dem einzelnen Patienten pathologisch aus In der gesamten Gruppe waren die voluntaren Augenbewegungen verwundbarer als die reflexiven. Nach dem Blickfolgetest und dem voluntaren Sackadentest ist der optovestibulare Test am besten geeignet, wenn man Patienten mit Hirnstammslesionen von Normalpersonen unterscheiden will

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C Wennmo
Department of Otorhinolaryngology
University Hospital
S 221 85 Lund
Sweden

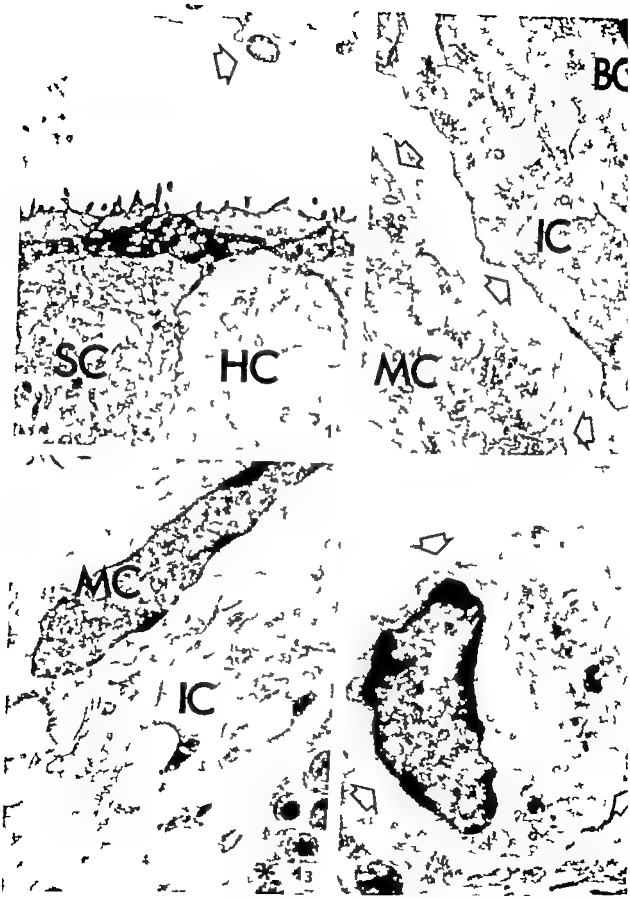
Fig 4 EM. Stria vascularis. Mature guinea pig. Organ culture for 7 days. 3700 and 42000 respectively (A) Poor preservation of specimen. Degenerative changes in marginal and intermediate cells: lipoid-like droplets (*L*) and electron-optically dense rounded structures (*arrow*). (B) Higher magnification of specimen in Fig. A. Myelin figures are indicated (*arrow*). Clumping of mitochondria?

Fig 1 Electron microscopy (EM). Crista ampullaris of mature CBA/CBA mouse cultured in vitro for 7 days. The hair cell (*HC*) reveals less electron-dense cytoplasm in contrast to the supporting cell (*SC*) with more intense staining and distended rough endoplasmic reticulum. The hair cell has lost its sensory hairs. Sensory hair fusion is indicated by an arrow 4800

Fig 2 EM. Newborn guinea pig. Cells of the stria vascularis (*MC* marginal cell *IC* intermediate cell *BC* basal cell) preserved in vitro for 3 days. Cytology is well preserved. The intercellular spaces have increased as compared with material taken for immediate fixation. Basal membranes outline the adjacent surfaces of marginal and intermediate cells (*arrow*). 13500

Fig 3 EM. Stria vascularis from mature guinea pig. Organ culture for 7 days. Electron optically pale regions occur in strial cells (lipoid-like material?). Electron optically dense material is found focally in some cells (*asterisks*). *During dissection of the specimen the stria vascularis and the hair cells became apposed to each other.* *HC* hair cell. One sensory hair is preserved in this section and the rootlet of another is visible. *MC* marginal cell *IC* intermediate cell 7400

Fig 5 EM. Blood vessel in the stria vascularis. CBA/CBA mouse. Organ culture for 6 days. Endothelial cells are swollen though not oedematous. Electron dense accumulations in the cytoplasm. Clumping of degenerating mitochondria. The basal membrane surrounds the blood vessel (*arrow*). 8500


BC
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1
MC
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3

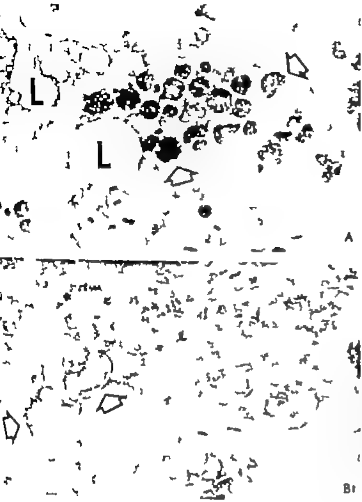

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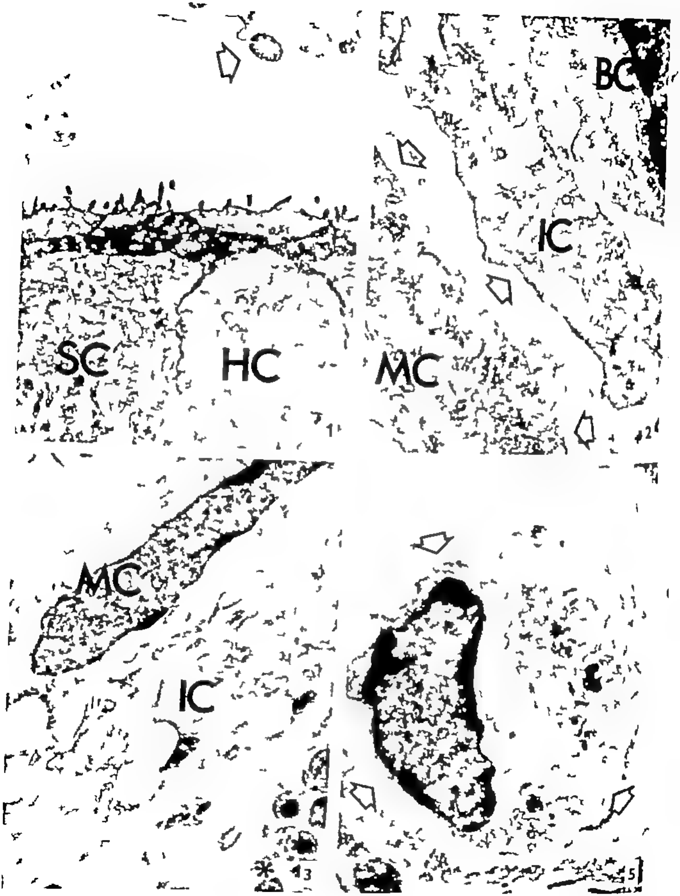
Fig 5 EM Blood essel in the stria vascularis, CBA/CBA mouse Organ culture for 6 days. Endothelial cells are often though not oedematous Electron dense accumulations in the cytoplasm. Clumping of degenerating mitochondria The basal membrane surrounds the blood vessel (*arrow*) 8 500.


BC
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SC
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1
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MC
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5

Fig. 9. EM. Dark cell of the newborn CBA/CBA mouse. Organ culture for 7 days. Normal morphology of both mitochondria and intercellular digitations. Basal membrane (*BM*). ×48 000.

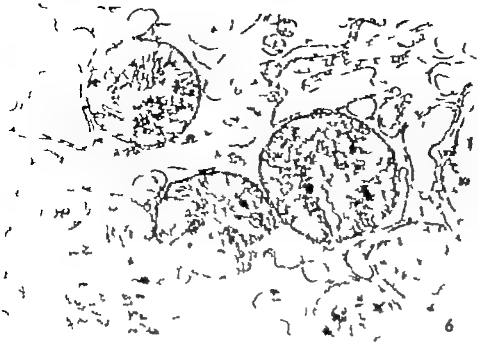
Fig. 10. EM. Dark cell of the mature CBA/CBA mouse. Organ culture for 7 days. Extremely distended rough endoplasmic reticulum (asterisks). ×26 000.

tions seemed less severe when explanting newborn inner ears as compared with mature ears. Otherwise there was no specific difference in cytological changes between newborn and mature organs. As regards the guinea pig, the cytological alterations seemed rather more severe than in the CBA/CBA mouse. Changes in fine structure, when present, were in principle the same for both types of animal.

Stria vascularis

Normal ultrastructure was preserved during the first 3–4 days in vitro though an increased vesiculation of cells was observed in many specimens. In the stria vascularis of the guinea pig a basal membrane adjacent to marginal and intermediate cells occurred also on the 3rd day in vitro (Fig. 2).

At the same time, accumulations of electron-dense material were identified in the cytoplasm of all three types of cells comprising the stria vascularis which at higher magnification were suspected to be composed of degenerating mitochondria (Figs. 3, 4 A and B). Similar degenerative changes also occurred in the endothelial cells of strial blood vessels (Fig. 5).



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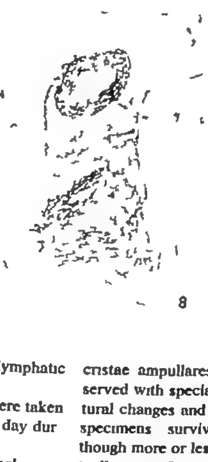

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Fig 6 EM Marginal cell of the stri vascularis Mature guinea pig O gan culture for 7 days. Degenera mitochondria showing cristae fra mentation and intramitochondri inclusion bodies ×28 500.
Fig 7 EM Specimen as in Fig. Here the mitochondria have lost their internal structure and appea swollen. ×27 000
Fig 8 EM Intermediate cell of t stria vascularis. Newborn CBA/CBA mouse Organ culture for 5 days Formation of myelin figures i the mitochondria. ×36 000.

so as to obtain a fairly enclosed endolymphatic space

Two inner ears from each group were taken for morphological investigation each day during 1–7 days in vitro

The details of the organ culture technique and morphological documentation have been described in detail by Anniko (1979)

RESULTS

Cells comprising the stria vascularis in the cochlea and the dark cell region around the cristae ampullares and the utricle were observed with special regard to early ultrastructural changes and survival of the cells In all specimens surviving cells were identified though more or less cytologically altered Actually very few cells of the secretory epithelium had disintegrated It was also possible to distinguish between hair cells and supporting tissue even when increasing culture time in vitro (Fig 1) The general configuration of the area comprising the secretory epithelium was preserved

In the CBA/CBA mouse cytological altera-

mitochondrial degeneration could be a secondary phenomenon visible also when the basal cell survival in vitro also becomes affected. As a comparison, the hair cells of the post-natal labyrinth are unable to preserve their specific structure in the extracorporal system for more than a few days (Anniko & Wersäll 1978) thereby being the weak point of the labyrinth in vitro, a fact that has often been observed in inner ear pathology (review: Hawkins, 1976; Wersäll et al. 1979).

During morphological maturation the normal stria vascularis has a basal membrane separating the marginal and intermediate cells (Kikuchi & Hilding 1966). This might help the cells in culture to demarcate towards the in vitro environment acting as an extra filter in addition to the plasma membrane. The surface regions of the marginal cells are normally regarded to possess highly active transport mechanisms which during organ culture may be sufficient to maintain cell integrity in this region of the cell directly facing the in vitro milieu.

A difference, although slight, was observed between newborn and mature animals with regard to cytological alterations in both vestibular and cochlear secretory epithelia. Tissues in newborn organs, especially those of the CBA/CBA mouse, had in general a well preserved fine structure throughout the culture period. According to Anniko & Wroblewski (1979) the formation of endolymph occurs 4–6 days post partum (CBA/CBA mouse). The difference in preservation of fine structure between newborn and mature tissues might therefore indicate that they have been explanted at different stages of physiological maturation.

In conclusion, the post-natal cells of the inner ear secretory epithelia are not dependent on specific endolymph environment to survive in vitro during their first week in culture. Whether or not this can influence their long term survival has to be the subject of further analysis. Cytological alterations in this study were seldom so severe that the cells passed beyond the point of no return in cell survival, thereby indicating a reversible morphological pathology.

ZUSAMMENFASSUNG

Neugeborene und voll entwickelte Innenohren des Meerschweinchens und der CBA/CBA Maus wurden in ein In-vitro-System eingepflanzt. Spezielle Aufmerksamkeit wurde den sekretorischen Regionen des Labyrinths geeignet, die überlebende Zellen während der einwöchigen Untersuchungsperiode in Organkultur aufwies. Sowohl Zellen der Stria vascularis als die dunklen Zellen der vestibulären Organe waren morphologisch ziemlich gut beibehalten. Degenerierende cytologische Veränderungen zeigten sich gegen Ende der Kulturperiode

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Matti Anniko, M.D.
Department of Oto-rhino-laryngology
Karolinska Sjukhuset
S-10401 Stockholm
Sweden

Fig 11 Light microscopy (LM) Crista ampullaris Organ culture for 7 days ×340 and ×270 respectively (A) Mature CBA/CBA mouse The dark cell area reveals small intracellular vesicles in many cells The general structure of the crista ampullaris is well preserved. Hair cells are indicated by arrows. (B) Mature guinea pig Dark cells show slight swelling with small intracellular vesicles Hair cells are indicated by arrows.

Cell junctions were in general well preserved

The mitochondrial fine structure could disintegrate in three different ways general cristae irregularities and fragmentation with accumulation of intramitochondrial inclusion bodies (Fig 6) cristae disintegration with electron-optically empty regions (Fig 7) and finally formation of myelin figures (Fig 8) though the latter was less frequent

Dark cell region of the crista ampullaris and the utricle

The cytological changes are similar to those observed in the cells of the stria vascularis but in general the dark cells appear better preserved (Fig 9) One characteristic of these cells is however the extreme distention dilatation of the rough endoplasmic reticulum (Fig 10) Degenerating mitochondria frequently revealed formation of myelin figures. The general preservation of the organs is good both in the guinea pig and in the CBA/CBA mouse (Fig 11 A and B)

DISCUSSION

Post natal cells of the secretory epithelium are according to the present study generally well preserved during the first week in vitro After 3–5 days in culture degenerative changes in cytological fine structure do occur however The functional importance of these changes is difficult to predict by morphological methods only A similar transformation of the fine structure in strial cells has been observed following administration of ethacrynic acid and was then correlated to changes in physiological activity impaired endocochlear potential and alterations in the ion composition of endolymph (Bosher 1977) In contrast to ethacrynic acid induced morphological (initially reversible) changes in the stria vascularis (Anniko 1978) no such changes can be observed in the dark cells comprising the vestibular secretory epithelium (Anniko unpublished observations) This is attributed to differences in cell physiology/function/activity between the two types of epithelium Furthermore the dark cells were better preserved in vitro than were cells of the stria vascularis

It is likely however that the cells comprising the secretory epithelia of the inner ear initially maintain—at least in part—their specific ion transport system and capacity to induce ionic gradients Later this specialized function might be gradually lost Morphological correlates to these assumed physiological alterations are unlikely except perhaps for the adjustment period of the cells until reaching a stage of basal cell survival/adaptation in vitro e g showing increased cell vesiculation

Ultrastructural identification of extensive

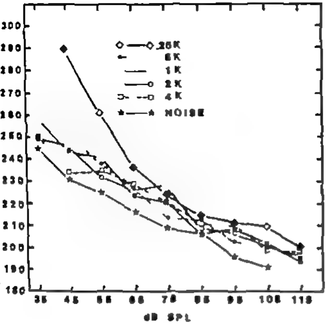

Fig 3 Reaction time (in msec) as function of frequency and intensity for the group of 10 listeners

apparent Only two subjects had individual slopes at 1000 Hz greater than 0.33 their slopes were 0 70 and 0 57 more in agreement with previous results These two subjects were experienced in magnitude estimation Two other experienced subjects however had low slopes of 0.33 and 0 16.

Equal-loudness contours were constructed from the group data. The reference frequency was the customary 1000 Hz, and the spacing at 1000 Hz was in 10 dB steps from 35–115 dB SPL An interpolation procedure was used to find the intensities at other frequencies for equal loudness to the 1000 Hz stimuli Our procedure used the power function formula log $Y = B \log X + \log A$ where Y is the geometric mean of the loudness judgments X is the sound pressure and B and $\log A$ are the slope and the intercept on the Y axis respectively for the best-fitting regression line Each frequency had its own unique *power function* that is each frequency had a unique slope and intercept The first step was to solve for Y in the regression formula for each intensity at 1000 Hz. Then for each of the different frequencies the obtained Y values for 1000 Hz were substituted into the regression formula for that frequency to solve for X the sound pressure Sound pressure was converted to dB SPL so that each contour represents the required dB SPL for equal loudness (Y) to the 1000 Hz reference

The equal-loudness contours (Fig 2) were in reasonable agreement with other loudness contours (Ross 1967 Schneider et al. 1972) Frequencies above and below 1000 Hz required greater intensity for equal loudness at the lower intensity levels (a slight dish shape) and as intensity increased the contours flattened slightly If lower intensities had been used the dish shape at low levels and the flattening at high levels would have been more pronounced Note that the intensities for 250, 500 and 000 Hz at 115 dB SPL are slightly above those actually presented to the subjects.

Reaction time

Reaction time decreased as intensity increased (Fig 3) with the greatest change being at the low intensities as was expected from the previous work of Stebbins (1960) and Pfingst et al (1975) At 35 dB SPL the lower frequencies

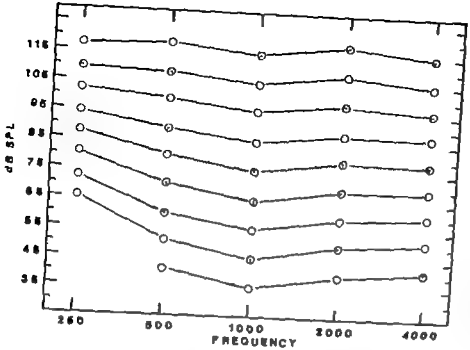

Fig 2 Equal loudness contours derived from magnitude estima data for the group of 10 listener

button when you are through Write down the number on your answer sheet The light will come on again upon pre sentation of the next tone

Try to make the ratios between the numbers you assign to the different tones correspond to the ratios between the loudness of the tones In other words try to make the numbers proportional to the loudness as you hear them For example if you called the first tone 20 and the second sounds five times as loud, call it 100 If it sounds one-half as loud say 10 if a fifth as loud say 4 etc Try not to worry about being consistent. Try to give an appropriate number to each tone regardless of what you remember that you called this tone before

The first magnitude estimation session was a practice session Data was then gathered in two additional sessions with all of the stimuli being presented once per session The order of the stimuli was random

RESULTS AND DISCUSSION

Loudness

Direct magnitude estimations were made to pure tone and noise stimuli presented ran domly over stimulus type and a range of inten sities Geometric means were computed a cross all subjects and power functions were obtained for each of the five frequencies and for the noise Power functions are linear corre lations performed on log-log transformed data and provide the best least squares fit to pro thetic (magnitude) data (Stevens 1975)

Power functions provided a good fit to ou magnitude estimation data Correlations be tween loudness and intensity were betwee 0 98 and 0 99 for all signals The rate of loud ness (slope) was relatively constant across sti mulus frequency (0.34 0 32 0 33 and 0.36 at octave frequencies from 500 to 4 000 Hz re spectively) Schneider et al (1972) found rela tively constant slopes above 400 Hz up to 2 500 Hz, the highest frequency in their experiment Our results indicate that the slopes continue to remain constant up to 4 000 Hz. Again in a greement with Schneider et al (1972) our 250 Hz condition showed a faster rate of loudness increase (0 44) than the higher frequencies The slope for broad-band noise was lower than for that of any of the tones (0 28)

The slopes for all stimuli for our group were low compared to those for other reported data (Marks 1974) The lowest previously report ed magnitude estimation slope for 1 000 Hz for example is 0 43 (Stevens & Guirao 1962) and our 1 000 Hz slope is 0 32 No explanation for this discrepancy other than the instability of beta weights and the possible presence of number bias by individual subjects is readily

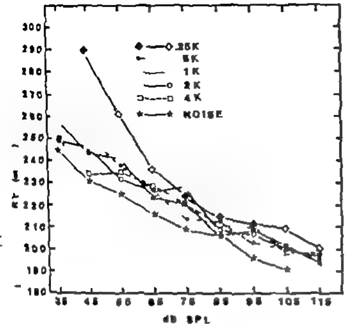

Fig. 3. Reaction time (in msec) as function of frequency and intensity for the group of 10 listeners

apparent. Only two subjects had individual slopes at 1000 Hz greater than 0.33: their slopes were 0.70 and 0.57, more in agreement with previous results. These two subjects were experienced in magnitude estimation. Two other experienced subjects, however, had low slopes of 0.33 and 0.16.

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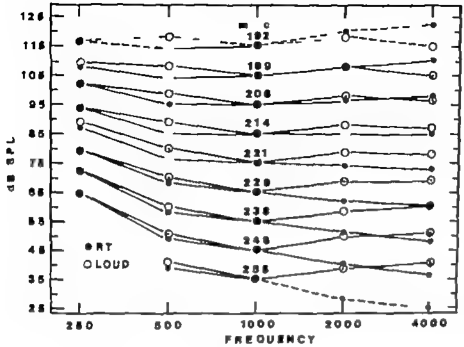

Fig 4 Equal-loudness contours (○) and equal-reaction time contours (●) as a function of frequency for the group of [illegible] listeners The parameter is reaction time (in msec). Extrapolated data are indicated by dashed lines

produced longer reaction times than did the higher frequencies but this frequency effect disappeared at higher intensities Reaction times were nearly always shorter for noise than for pure tones Using power functions fit to the 1000 Hz reaction time curve (R=0 98) and to the noise reaction time curve (R=0 99) interpolations were made to determine the sound pressure in decibels necessary to equate the reaction time for noise to that for the 1000 Hz signal At 35 dB SPL the noise required 14 dB less sound pressure than the 1000 Hz signal to produce equal-reaction time and at 105 dB SPL the noise required 10 dB less sound pressure than the 1000 Hz signal

Reaction time and loudness

The group reaction time data were entered into a computer curve-fit program for linear power exponential and parabolic functions Power curves consistently provided the best fit to the data which is in agreement with Moody (1970) Correlations between reaction time and intensity were [illegible] 94 for 250 Hz [illegible] 98 for 500–4000 Hz and [illegible] 99 for noise Equal latency curves were constructed using the same procedure as described earlier for equal-loudness curves

Fig 4 shows a comparison between the equal-latency and equal-loudness contours. These contours were in agreement (5 [illegible] or less) between 75–105 dB SPL. At lower intensities the contours were affected by the relatively fast reaction times of 2000 and 4000 Hz. The extrapolated results for equal-latency of reaction time at 115 dB are most likely questionable since the power function was fit to our obtained reaction times at levels from 35 to 115 dB SPL. However the latency curve plateaus at high intensities and as our extrapolated results do not take this flattening of the slope into account the extrapolations are no doubt inaccurate

Since each subject contributed just four reaction time to the overall average [1] and since intra subject reaction time variability typically

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[1] The admittedly small number of individual judgements (4) for each datum point was due to the fact that simultaneous middle ear reflex measurements were made throughout the reaction time portion of this experiment. Additional stimulus presentations were precluded by the continuing discomfort of the probe mechanism over the duration of the experimental session The relation between loudness and middle ear muscle activity has been described elsewhere (paper presented to the American Audiology Society Miami 1977 Abstract published in *Corti's Organ* 3 No 1 4–5)

is large (Moody Beecher and Stebbins 1976) we decided to measure the reaction time of one subject in depth The loudness contours for the group and for the one subject (each reaction time measure based on 20 stimulus presentations) were nearly coincidental

Moody (1970 1973) compared latency contours from monkeys (Stebbins 1966) with loudness contours from humans (Fletcher & Munson 1933) Pfingst et al (1975) made the loudness and latency time contour comparison for one human subject Equivalence of loudness and reaction time latency have been assumed because of the similar shape of the contours. The present data provide even stronger evidence that reaction time is a measure of loudness Although group equal-latency time and equal-loudness contours show some dissimilarities each individual in the group contributed only four reaction times to each mean datum point. Obtaining more thorough reaction time measures on an individual subject resulted in much better agreement between latency and loudness contours

In conclusion loudness perception can be inferred from auditory reaction time data Unfortunately reaction time requires extensive prior practice and many measurements for each data point in order to obtain stable results Thus reaction time measurement can be a valuable research tool but has limited use for clinical testing

ACKNOWLEDGEMENT

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ZUSAMMENFASSUNG

Kurven gleicher Lautheit für Töne von 250–4 000 Hz wurden von magnitude estimation judgment an zehn Personen mit normalem Gehör gebildet Kurven gleicher Latenz wurden von auditory reaction time measurements gebildet. Kurven gleicher Lautheit stimmten mit denen früherer Forschungen gut überein und die Kurven gleicher Latenz stimmten mit den Kurven gleicher Lautheit auch gut überein

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John F Brandt, Ph D
Bureau of Child Research Labs
1043 Indiana
Lawrence
Kansas 66045
USA

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IMMEDIATE ALTERATIONS IN THE IMPULSE NOISE EXPOSED ORGAN OF CORTI OF THE GUINEA PIG

Ch Meyer and M Biedermann

From the Institute of Anatomy and Institute of Physiology Friedrich Schiller University of Jena DDR

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Abstract Cochlear microphonics and morphological changes in the organ of Corti were examined in guinea pigs exposed to 1 or 10 impulses of 164 dB SPL. Immediately after noise exposure the CM amplitudes of frequencies between 500 and 10000 Hz declined severely with an accelerating tendency during the next 2 hours The decrease in CM in the two groups differed insignificantly 10 minutes after impulse exposure whole-mount specimens showed mainly swollen and translocated nuclei of outer hair cells After 2 hours we found large quantities of pyknotic nuclei karyorrhexis, and gaps in the normal OHC pattern Despite minor numerical differences in injured cells of either group exposure to 10 impulses caused a greater degree of irreversible alteration Altogether altered hair cells were only localized in the basal and lower second turn and their numbers were relatively small This stands in contrast to the severe decrease of CM from low to high frequencies Hair cell injury and additional mechanical lesion of the reticular membrane caused many sites of leakage allowing interchange of endo- and perilymph. This effect is considered to be the main cause of functional damage indicated by rapid CM decline.

Recent studies concerned with lesions of the cochlea previously exposed to impulse noise resulted in various morphological and physiological findings (Poche et al 1969 Jordan et al 1973 Henderson et al 1974) Due to the interval between sound exposure and commencement of the investigation as well as the relative high number of impulses applied these findings were inconclusive as regards the mechanism(s) of alteration For that reason we decided to study immediate alterations of the guinea pig cochlea by physiological and morphological methods The present paper reports on the findings of these studies

MATERIAL AND METHODS

The studies were carried out on 46 young guinea pigs of 140–280 g body weight. All of the animals had a positive ear reflex according to Preyer Under general anaesthesia with urethane (1 5 g urethane per kg body weight) injected intraperitoneally the tympanic bulla had been dissected from occipital so as to prevent lesions of the tympanic membrane and the ossicular chain

Cochlear microphonic potentials (CM) were registered from the margin of the round window by means of a silver–silver chloride electrode for frequencies of 500 1 000 2 000 3 150 5 000 8 000 and 10 000 Hz (produced by a sinus generator) Filtering magnification and evaluation of CM were carried out with an audio-frequency spectrometer type 2109 (Brüel & Kjær Denmark) Following the measurement of primary CM amplitudes the guinea pigs were exposed to 1 or 10 impulses at intervals of 15 s Impulse noise was produced by means of a spark noise generator (Zentralwerkstatt für Forschung und Entwicklung Bereich Medizin FSU Jena) of an intensity of 164 dB SPL with mean duration of <0 1 ms Following the exposure to impulse noise CM were registered for the afore mentioned frequencies again either only once immediately or for a period up to 2 hours

Immediately following the last measurement of CM (5–10 min or 2 hours after impulse

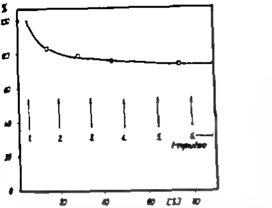

Fig 1 Decline in CM of the guinea pig during exposure to single impulses of 164 dB SPL. Arrows mark the time of application of single impulses. Registration of CM took place 10 after each impulse exposure for the frequency of 3 150 Hz, 80 dB SPL (=10). *Abscissae* time *ordinate* CM % of the original amplitudes

noise exposition) the cochleae were fixed first by perilymphatic perfusion in vivo and subsequently after decapitation of the animals and dissection of the cochleae by means of immersion. The fixative applied consisted of % paraformaldehyde 2% glutaraldehyde in 0.05 M cacodylate buffer pH 7.4 Whole mount specimens comprising the basilar membrane with the tympanic cell layer and the organ of Corti were stained with gallocyanine–chromalum (Pearse 1968) Hair cells and supporting cells of the organ of Corti were examined by light microscopy along the entire length of the basilar membrane and alterations in the normal cellular distribution pattern were registered both quantitatively and qualitatively This technique however did not allow for a constant and complete preparation of the first 1– mm of the basal turn

RESULTS

Electrophysiological findings

Single impulses of 164 dB SPL always cause a reduction in CM amplitudes. Fig 1 demonstrates for a frequency of 3 150 Hz that the first impulse of a series causes the greatest reduction in CM amplitudes Effects of the subsequent impulses have also been registered, though their contribution to the reduction in CM decreases constantly

Immediately after the exposure to 10 impulses of 164 dB SPL the reduction in CM for low and high frequencies amounts to 27–36% of the original amplitudes (Table I) One can see a somewhat more pronounced diminution in CM at higher frequencies. Furthermore Table I shows how CM decreases during further registration Two hours after exposure to impulse noise the reduction has amounted to 60–79% of original amplitudes Now in particular the frequencies 3 150 and 5 000 Hz were reduced

Immediately after exposure to a single impulse of 164 dB SPL the CM amplitudes for frequencies from 500–10 000 Hz decreased to about 13–22% of the original amplitudes (Table II) By the end of the registration 2 hours after impulse noise exposure one can find a further reduction in CM amplitudes which has now attained 46–70% of the original amplitudes

Control animals not exposed to impulse

Table I *Mean reduction of CM-amplitudes of 17 guinea pigs exposed to 10 impulses of 164 dB SPL In % of the original amplitudes*

% amplitudes following impulse exposition	Frequencies tested						
	500	1 000	000	3 150	5 000	8 000	10 000 Hz
Immediately after impulse exposure	71	69	73	71	67	64	66
After 1 hour	49	53	51	45	43	5*	46*
After hours	3**	31	3**	4	1	34*	35*

Mean alues of lower number (9–14) of single alues

Table II *Mean reduction of CM amplitudes of 14 guinea pigs following the exposure to a single impulse of 164 dB SPL in % of the original amplitudes*

% amplitudes following impulse exposure	Frequencies tested						
	500	1 000	2 000	3 150	5 000	8 000	10 000 Hz
Immediately after impulse exposure	87	83	[illegible]	80	83	79	83
After 1 hour	65	62	[illegible]	47	63	45	47
After 2 hours	54	41	43	30	47	32	45

noise did not exhibit any alteration in the CM amplitudes over a period of 2 hours

Morphological findings

In the entire series of specimens studied the organs of Corti displayed nuclear changes such as (*a*) swelling of nuclei (*b*) pyknotic nuclei (*c*) karyorrhexis and (*d*) missing nuclei (or loss of the complete cell)

Alterations (*a–c*) were very often accompanied by a translocation of the nuclei Almost all of these alterations could be found in the first turn or in the lower half of the second turn and they were mostly restricted to OHC Rarely enlarged nuclei of IHC have been detected Third row OHC in most of the specimens were the most frequently altered Very often single or small groups of altered nuclei were localized in between apparently normal HC Regions of severely damaged nuclei sometimes displayed additional ruptures of pillar cells or even complete loss of pillar cells Those regions could demonstrate a profound disintegration of the organ of Corti such as to render the recognition of the normal pattern of cells impossible Although localization of alterations was found constantly in the first and the lower second turn the intensity of individual lesions varied considerably

Some 5–10 minutes following the exposure to 10 impulses swollen nuclei of OHC prevailed They were more numerous in the basal turn except in one out of 9 cochleae which exhibited a greater number of nuclear swellings in the second turn With the exception of one cochlea pyknotic nuclei were rarely detected Karyorrhexis could not be seen The numbers of missing nuclei of OHC were clearly smaller than those of swollen nuclei However they were significantly greater than those of non-exposed animals The total numbers of changed nuclei of OHC varied between 68 and 476

Two hours following the exposure to 10 impulses the total number of changed OHC had not increased There was a remarkable increase in alterations in the lower second turn in some of the cochleae studied The ratio of various types of nuclear changes had shifted. There were roughly equal numbers of swollen nuclei and losses of OHC nuclei and increasing numbers of pyknotic nuclei and karyor

Table III *Percentage of nuclear alterations of outer hair cells*

	Missing nuclei (%)	Swollen nuclei (%)	Pyknosis (%)	Karyorrhexis (%)
5–10 min after 10 impulses	9.2	84 8	3 0	–
2 hours after 10 impulses	44 0	38 4	11.5	5 8
5–10 min after only one impulse	13 6	86.0	0 4	–
2 hours after only one impulse	19 9	52.4	19.3	8 4

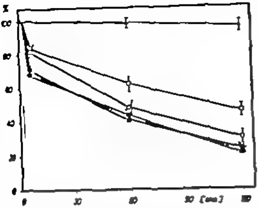

Fig 2 Comparison of the reduction in CM following exposure to 1 or 10 impulses (=13 or 17 respectively) of 164 dB SPL for period of 2 hours and frequencies of 3 150 and 5 000 Hz, 80 dB SPL. Arrow marks the exposure to impulses ○—○ 3 150 Hz, 1 impulse ●—● 3 150 Hz, 10 impulses, □—□ 5 000 Hz, 1 impulse ■—■ 5 000 Hz, 10 impulses, △—△ controls without exposure to impulses (10). *Abscissae* time [min]; *ordinates* CM % of the original amplitudes

rhexis had been detected In this group of animals swollen nuclei with irregular profiles were now more numerous and some of them exhibited coarse intensely stained chromatin particles In some confined areas the normal distribution pattern of nuclei was lost and had been replaced by an irregular distribution of altered nuclei with gaps in between. The total number of changed nuclei varied from 57 to 888 per animal

Alterations following a few minutes after exposure to one impulse were fairly similar to those after the application of 10 impulses In 9 cochleae studied swollen nuclei were prevalent and the loss of only a small number of OHC nuclei could be detected Pyknotic nuclei and karyorrhexis were almost completely absent. In comparison with the 10-impulse group there was only a small reduction of changed OHC The total number varied between 66 and 351 nuclei per animal

Two hours following the exposure to one impulse the light microscopic examination revealed mainly swollen nuclei pyknosis, and karyorrhexis and in addition a somewhat larger number of lost OHC Disintegration of the organ of Corti as was found following the exposure to 10 impulses was not detectable in any of the 9 cochleae studied However small areas of about 5–10 OHC displayed almost all types of nuclear changes often associated with internal translocation which gave a more or less irregular pattern. The total number of changed HC was about the same as after 10 minutes of sound exposure and it amounted to 44 and 417 cells per animal

DISCUSSION

The present electrophysiological findings following the exposure to 1 or 10 impulses proved an immediate reduction of CM which proceeded considerably during the ensuing 2 hours There is no indication of any recovery during that period. This is in accordance with earlier results published by Biedermann et al (1977) In comparison with animals exposed to one impulse the CM recorded at 3 150 and 5 000 Hz respectively were only slightly more reduced following the exposure to 10 impulses (Fig. 2) According to the findings displayed in Fig. 1 the first impulse applied results in the greatest reduction of CM potentials And this casts light on the role the first impulse may play in the mechanism of lesions of the organ of Corti

Consistent with these electrophysiological results morphological studies could demonstrate only small differences between the 1 and 10-impulse group 2 hours following the noise exposure Due to the confinement of the gallocyanine–chromalum staining to nuclei the morphological examination is almost entirely restricted to the nucleus as the sole indicator of lesions. Furthermore one could see ruptures of the reticular membrane and the detachment of pillar cells. Fine structural alterations such as plasmalemmal lesions swelling or fusion of receptor cell hairs, are not demonstrated by this method However the findings indicate a nuclear swelling as the first change which can be seen only 10 minutes after exposure to impulse noise Swelling of the hair

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2 hours after only one impulse	19 9	52 4	19.3	8.4

CM. The decline in potentials had been registered in all frequencies tested despite a clear-cut confinement of morphological alterations to the first and the lower second turn. According to the electron microscopic studies of Geyer et al. (1978) the considerable decline in CM in all frequencies tested is caused primarily by ruptures of the reticular membrane. Both ruptures and loss of cells result in gaps in the cellular construction of the organ of Corti and they allow for the exchange of endolymph and perilymph and in this way result in the loss of the positive endolymphatic potential which is considered a prerequisite for the normal functioning of HC. Alterations in the nuclei of IHC are a rare event. Presumably these cells are more resistant towards mechanical effects because they are more completely enveloped by supporting cells and possibly even more significant, they occupy a position on the basilar membrane which is less dislocated by oscillations during sound exposure. Functional alterations due to gaps in the cellular barrier of the cochlear duct will affect IHC as well as OHC.

The present studies on cochlear lesions due to high energy impulse noise demonstrated an immediate decrease in CM associated with morphological alterations of OHC, an effect which increased over a period of 2 hours. The relatively small proportion of HC which were affected as well as their localization in the first and the lower second turn is apparently at variance with the electrophysiological findings. Presumably this discrepancy of physiological and morphological findings is easily explained by ruptures in the reticular membrane and ensuing interchange of endolymph and perilymph (Geyer et al. 1978, Schmidt et al. 1978). HC exhibiting plasmalemmal defects are thought to cause gaps in the normal tight barrier which is built up to separate scala media and scala tympani. According to Bohne (1976) defects of this kind may get repaired within days or weeks according to their extent. The repair of the endolymphatic barrier is considered a prerequisite for normal functional conditions and it should be followed by the buildup of normal ional gradients which can be registered between endolymph and perilymph. However, healing of these defects will not make good the losses of HC which therefore must result in a permanent lesion of organ of Corti.

ZUSAMMENFASSUNG

Meerschweinchen wurden mit 1 bzw. 10 Impulsen von 164 dB SPL beschallt und danach das Verhalten der cochleären Mikrophonpotentiale (CM) sowie morphologische Veränderungen des Haarzellmusters untersucht. In einem Frequenzbereich zwischen 500 und 10000 Hz zeigen die CM unmittelbar nach der Schallbelastung eine deutliche Minderung mit weiter fallender Tendenz in den folgenden Stunden. Die CM-Abnahmen beider Gruppen unterscheiden sich nur unwesentlich. Sofort nach der Beschallung überwiegen im Häutchenpräparat geschwollene und meist verlagerte Kerne der äußeren Haarzellen (ÄHZ). Nach 2 Stunden treten vermehrt Pyknosen und Karyorrhexis sowie Lücken im normalen ÄHZ-Muster auf. Obgleich die Zahlen veränderter Zellkerne in beiden Versuchsserien sich nur wenig unterscheiden, verursachen 10 Impulse einen höheren Prozentsatz an irreversiblen Schädigungen. Insgesamt erscheint die Anzahl geschädigter ÄHZ, die nur auf die basale und untere 2. Windung beschränkt sind, relativ klein, verglichen mit dem starken Abfall der CM-Amplituden in allen geprüften Frequenzbereichen. Als Hauptursache für den schweren funktionellen Schaden werden daher neben den HZ-Veränderungen die Durchmischung von Endo- und Perilymphe infolge Leckstellen in der Retikularmembran angesehen.

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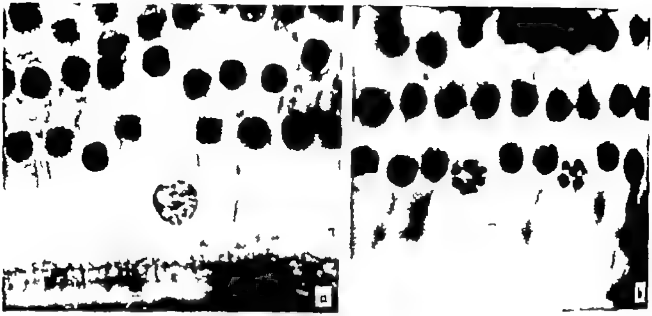

Fig 3 Swollen nuclei of first row outer hair cells. (*a*) 10 minutes after 1 impulse (*b*) 2 hours after 10 impulses transitional stage to karyorrhexis.

cell nuclei following an exposure to lower—physiological—sound intensity had been described previously (Neubert & Wüstenfeld 1955 Beck 1955 1956 Wüstenfeld 1958) and according to these authors they were considered reversible The majority of swollen nuclei of impulse noise exposed cochleae however appeared to be non physiologically altered Many of them had been distorted and they displayed irregular distribution and some kind of condensation of chromatin already 5–10 minutes after sound exposure and even more 2 hours after exposure There is a continuous line of development which can be followed from pathological swellings of nuclei to nucleopyknosis and karyorrhexis Both of these are particularly numerous 2 hours after sound exposure It is not clear however whether or not some pyknotic nuclei which had been detected already 5–10 minutes after exposure had passed a stage of swelling In any case nucleopyknosis karyorrhexis and total loss of OHC were seen in large amounts only 2 hours after high energy sound exposure There is only a slight increase in the total number of changed OHC during 2 hours after sound exposure but the rates of various types of nuclear alterations had shifted Hence one may conclude that at least some swollen nuclei may develop to pyknosis and karyorrhexis or the particular HC may be lost completely Confined disintegrated areas whose OHC were already severely altered or even degraded were seen only after the exposure to 10 high energy impulses

Microscopic examination of the OHC and their pattern of alteration showed that the third row OHC nuclei were changed more often than nuclei of the first or second row of OHC Quantitative comparison of individual findings once more demonstrated the great variability of alterations and furthermore disproved the Gaussian distribution curve of alterations i e it invalidated any statistical calculation For that reason the sign-test according to van der Waerden has been employed and by this means a difference between altered 3rd row hair cells has been demonstrated in comparison with alterations of first and second row OHC with a level of error of about 1% However in one series of experiments it was only 2.5%

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CM. The decline in potentials had been registered at all frequencies tested despite a clear-cut confinement of morphological alterations to the first and the lower second turn. According to the electron microscopic studies of Geyer et al. (1978) the considerable decline in CM at all frequencies tested is caused primarily by ruptures of the reticular membrane. Both ruptures and loss of cells result in gaps in the cellular construction of the organ of Corti and they allow for the exchange of endolymph and perilymph and in this way result in the loss of the positive endolymphatic potential which is considered a prerequisite for the normal functioning of HC. Alterations in the nuclei of IHC are a rare event. Presumably these cells are more resistant towards mechanical effects because they are more completely enveloped by supporting cells and, possibly even more significant, they occupy a position on the basilar membrane which is less dislocated by oscillations during sound exposure. Functional alterations due to gaps in the cellular barrier of the cochlear duct will affect IHC as well as OHC.

The present studies on cochlear lesions due to high energy impulse noise demonstrated an immediate decrease in CM associated with morphological alterations of OHC, an effect which increased over a period of 2 hours. The relatively small proportion of HC which were affected, as well as their localization in the first and the lower second turn, is apparently at variance with the electrophysiological findings. Presumably this discrepancy of physiological and morphological findings is easily explained by ruptures in the reticular membrane and ensuing interchange of endolymph and perilymph (Geyer et al. 1978, Schmidt et al. 1978). HC exhibiting plasmalemmal defects are thought to cause gaps in the normal tight barrier which is built up to separate scala media and scala tympani. According to Bohne (1976) defects of this kind may get repaired within days or weeks according to their extent. The repair of the endolymphatic barrier is considered a prerequisite for normal functional conditions and it should be followed by the buildup of normal ional gradients which can be registered between endolymph and perilymph. However, healing of these defects will not make good the losses of HC which therefore must result in a permanent lesion of organ of Corti.

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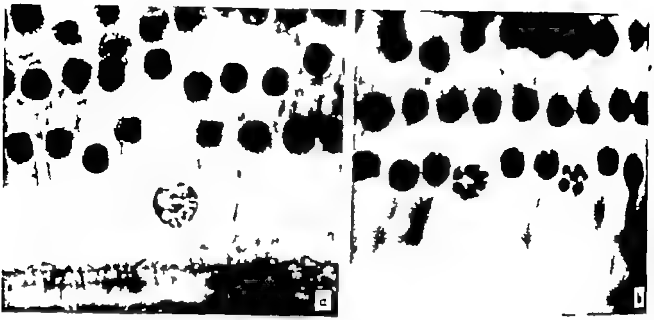

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TEMPORAL BONE FINDINGS IN A CASE OF SUDDEN DEAFNESS AND RELAPSING POLYCHONDRITIS

Tomoyuki Hoshino Tetsuo Ishii Akira Kodama[2] and Isao Kato[3]

Departments of Otolaryngology, [1]Hamamatsu University School of Medicine, [2]Teikyo University School of Medicine and [3]Yamagata University School of Medicine

(Received January 3 1980)

act. Light microscopic examination was made of the temporal bone from 57-year-old female who devel- sudden total hearing loss in both ears 1 year before h. The patient had suffered from relapsing poly- ndritis for 11 years prior to death. Pathological changes e compatible with known viral endolymphatic laby- thitis. Slight ossification and fibrous tissue proliferation e perilymphatic space seem to have been caused by read of infection from the middle ear. Susceptibility ural infection in the labyrinth in patients with long- asting illness is discussed.

e scanning electron microscopic findings of suddenly deafened inner ear occurring in a se of relapsing polychondritis were reported y the authors in a previous issue of this jour al (Hoshino et al. 1978). While that study ealt only with the left ear the present paper eports on the light microscopic findings in he contralateral ear of the same patient. The indings observed in this temporal bone had athological changes suggestive of the viral endolymphatic labyrinthitis found in the left ear.

The temporal bone pathologies found in this case shed light on a group of profound deaf nesses of unknown etiology namely sudden profound deafnesses occurring during the course of chronic devastating illnesses.

CASE HISTORY

A 56-year-old female experienced profound sensorineural deafness in both ears during the course of relapsing polychondritis. Complete hearing loss and vestibular disorders occurred suddenly in the left ear in September 1976 and the same episode occurred in the right ear on the following day. Steroids were continuously administered from December 1976 and aza thioprine from March 1977 in varying doses. The patient died of gastrointestinal hemor rhage in October 1977 1 year after these aural episodes. A detailed clinical history was given in the previous report.

The temporal bones were removed during autopsy 3 hours after death. The right ear was fixed in 10% formalin, decalcified in 5% tri- chloracetic acid and embedded in celloidin. The bone was serially sectioned in the hori zontal plane and every tenth section was stained with hematoxylin and eosin.

Temporal bone findings

Light microscope observation of the right ear showed a well-pneumatized bone. No change was found in the outer ear canal. The middle ear mucosa showed moderate changes of ac tive infection such as mucosal thickening, in- filtration of polymorphonuclear cells and exudate in the middle ear cleft. The round window membrane and the mucous lining of the round window niche showed thickening of the mucosa.

In the inner ear fibrous tissue proliferation and accumulation of proteinoids were found in the scala tympani near the thickened round window membrane (Fig. 1). Bony obstruction of the lateral semicircular canal at the non- ampullated limb and scanty proliferation o

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D Ch Meyer
Inst of Anatomy
Friedrich Schiller Universität
DDR-69 JENA

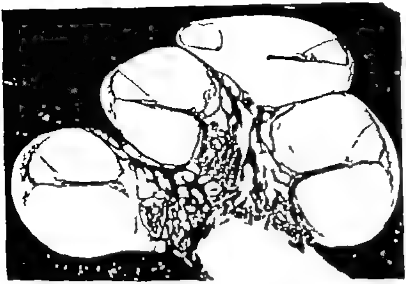

Fig. 3. Mid-modiolar view of the cochlea. Except for marked degeneration of the organ of Corti and patchy atrophy of the stria vascularis, no remarkable changes are to be seen in the cochlear duct or the spiral ganglion cells.

layer of flat cells. No innervating nerve fibres were found in the subepithelial connective tissue which was infiltrated by a thin homogeneously eosin-stained substance (Fig. 4B). In the utricle a moderate decrease of both sensory cells and nerve fibres was found (Fig. 4A). The sensory epithelia of the crista ampullaris of the three semicircular canals showed

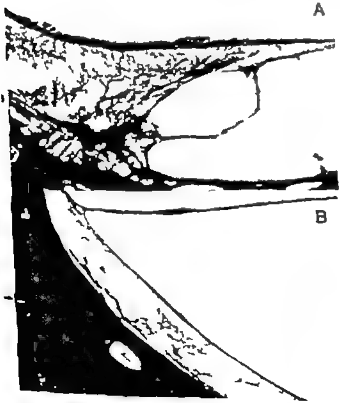

Fig. 4. Utricular and saccular maculae. Sensory epithelia were transformed into flat cuboidal cells in both the utricular (A) and saccular (B) macula. Dendritic nerve fibres were found only i the utricular macula.

Fig 1 Proliferation of fibrous tissue in the hook portion Round window membrane was thickened in continuity with inflammatory thickened mucous lining of the round window niche Note patchy degeneration of spiral ligament cells.

bony tissue and fibrous tissue in the perilymphatic space of the posterior semicircular canal were also found No tissue growth was found in other peri and endolymphatic spaces

Throughout the cochlea the organ of Corti was found to have degenerated It was compressed into a low mound and few of the outer and inner hair cells were discernible The tectorial membrane was covered by a layer of cells and adhered to the reticular lamina in the middle turn (Fig 2) or tucked onto the limbus or into the inner sulcus in other places The spiral limbus and spiral ligament seemed to be normal at all sites except the hook portion where there was a patchy absence of the spiral ligament cells Spiral ganglion cells and their axonic and dendritic processes were well preserved Reissner s membrane was in its normal position The stria vascularis showed patchy atrophy sporadically in every turn, but no granulomatous lesions were found (Fig 3)

No collapse or hydrops were found in the otolithic organs The utriculo-endolymphatic valve was seen to be wide open Sensory epithelia showed marked degeneration in both the utricular and saccular maculae In the saccule the macula was composed only of a

Fig 2 Organ of Corti in the middle turn Encapsulated tectorial membrane adhering to the degenerated organ of Corti

marked degenerative changes in the spiral ligament and spiral limbus suggestive of vascular changes were not observed in this temporal bone. Hence two different causative factors, virus and middle ear infection, seemed to have affected the same inner ear.

The marked degeneration seen in the superior portion of the vestibule of this left temporal bone is similar to the finding in the right ear. These vast degenerations are not common findings in the known histologies of viral endolymphatic labyrinthitis which mostly showed a Scheibe-type cochleosaccular degeneration with the exception of some measles cases (Lindsay & Hemenway 1954, Schuknecht 1974). Though the sensory epithelia showed similar degeneration in both the superior and inferior portions of the vestibule, there still remained a tendency of Scheibe type degeneration in the distribution of remaining dendritic nerve fibres innervating those endorgans.

The damage caused by any disease is modified by the virulence of causative factors on the one hand and the resistance of the host on the other. Recent advances in immunology are rapidly revealing how various factors effect changes in immunity. Autoimmune diseases, relapsing polychondritis being one of them, are known to have certain immunodeficiencies. Changes in immunity may have had some bearing on the vast divergence of inner ear changes in the two ears of the present case. Of 40 relapsing polychondritis cases reported by Cody et al. (1971), at least ? cases showed a profound deafness similar to the present one. Immunodeficiencies such as are found in autoimmune diseases or in protracted devastating diseases being treated with immunosupressants or steroids may have a higher susceptibility to viral labyrinthitis compared with the healthy ones (Dale & Petersdorf 1973).

ZUSAMMENFASSUNG

Eine mikroskopische Untersuchung über das rechte Schläfenbein einer 57jährigen Patientin wurde durchgeführt, die seit einem Jahr vor ihrem Tode einen plötzlichen Hörsturz auf ihren beiden Ohren bekommen hatte. Ferner war diese Patientin anderthalb Jahre lang an Relapsing polychondritis erkrankt. Der pathologische Befund im Ductus cochlearis erwies sich als derselbe wie der der bekannten Virusinfektion im Innenohr. Eine geringe Ossifikation und eine Proliferation des fibrösen Gewebes im perilymphatischen Raum schienen durch die Einwirkung der Mittelohrentzündung verursacht worden zu sein. Die Anfälligkeit für die Virusinfektion im Innenohr bei dem langfristig erkrankten Patienten wurde erörtert.

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T. Hoshino, M.D.
Dept. of Otolaryngology
Hamamatsu Univ. School of Medicine
3600 Handa-cho
Hamamatsu 431-31 Japan

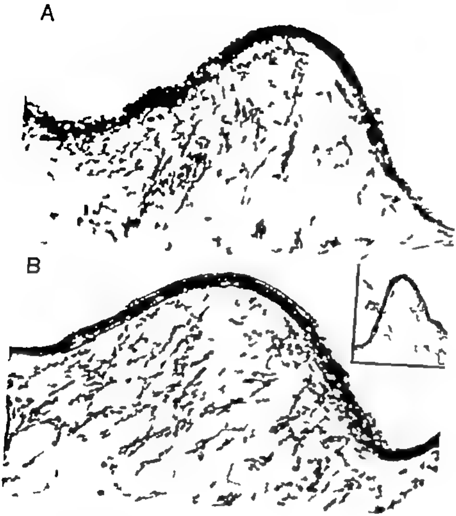

Fig 5 Ampullary cristae of posterior (A) and lateral (B) semicircular canal Sensory epithelia are transformed into a layer of cuboidal cells Scanty dendritic fibres still remain *Inset* An abnormal cupula (*arrow*) on the lateral semicircular crista

a marked degeneration as being covered by a layer of cells (Fig 5) A shrunken cupula adhering to the lateral semicircular canal crista showed an abnormal texture suggesting the degeneration of the cupula itself (Fig 5 inset) Innervating nerve fibres however seemed to be moderately preserved in all semicircular canals The endolymphatic duct and sac seemed normal

The eighth nerve and facial nerve trunks were pulled out during autopsy Cut surfaces of the small arteries in the fundus of the internal auditory canal showed thickening of the tunica media but no obstruction of the lumen

COMMENTS

The temporal bone in the present case showed inner ear findings similar to those previously reported in viral endolabyrinthitis (Lindsay 1967) namely encapsulated and dislocated tectorial membrane degeneration of the organ of Corti but good preservation of the neural elements compared with the marked change in the sensory epithelia

Findings for the right temporal bone were similar to those reported previously for the left ear except for a partial ossification of the posterior and lateral semicircular canals. Tissue proliferations have often been noted in inner ear injury caused by blood circulatory disturbances (Gussen 1976) As in the present case such changes were confined to the vicinity of the round window membrane which showed thickening by active infection of the mucosa, the ossification in the semicircular canals and proliferation of the fibrous tissue in the scala tympani of the lower basal turn seem to have been caused by spreading of the infection from the middle ear Furthermore

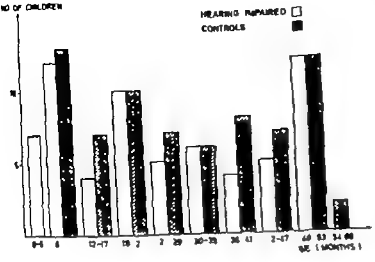

Fig. 1. The numbers of children in the various age groups. White columns indicate the hearing-impaired children, hatched columns indicate the control group.

They were all suffering from congenital hearing loss. As a control group (3) served 72 children with a median age of 29 months (range 6–60) without clinical symptoms of hearing loss. The majority of the latter group had been admitted for adenotonsillectomy but no routine audiometric examination was performed. Controls to group (1) were not included according to the serological method applied (see below).

METHODS

In groups (1) and (2) the congenital sensorineural hearing loss was demonstrated by various audiological procedures according to the age of the patients and their ability to cooperate in general audiometric procedures. When reliable psychoacoustic thresholds were unobtainable, elevated stapedial reflex thresholds elicited by contralateral stimulation by means of a Madsen impedance audiometer ZO 70 or 72, were used to estimate the hearing ability. Alternatively, the hearing loss was demonstrated by cortical evoked response audiometry and/or electrocochleography (Salomon 1977, Elberling, 1977).

Serum was collected from 8 patients in group (1) and tested for rubella IgM antibodies after separation of the immunoglobulins by rate zonal ultracentrifugation (Vesikari & Vaheri 1968, Vejtorp & Mansa 1979). Capillary blood from the remaining patients was collected on filter paper and tested for rubella haemagglutination inhibition (HI) antibodies as previously described (Leerhøy 1968). Children with rubella HI antibody titres of 1:10 or greater were regarded as seropositive and those with titres of less than 1:10 as seronegative.

Details of the maternal and family history, birth weight, condition of the child at birth and the presence of other defects were obtained from the clinical records on all the patients with hearing loss. Furthermore, when possible, information about postnatal rubella infection was included in the clinical records.

The results were analysed by the Mann-Whitney U-test and the χ-square test. The level of significance was set at 0.05.

RESULTS

In group (1) the serum samples from 5 patients with hearing loss contained rubella IgM antibodies. Capillary blood collected from 1 patient at the age of 5 and 12 months contained HI antibodies without any significant difference of the titres in the samples, whereas an-

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CONGENITAL HEARING LOSS AND RUBELLA INFECTION

A. Parving, M. Vejtorp, K. Møller and J. Hartvig Jensen

From the Audiology Clinic, Gentofte University Hospital, Rubella Department, Statens Seruminstitut, Copenhagen, ENT Department, Copenhagen University Hospital, Hvidovre, and the State Hearing Centres of Copenhagen, Odense and Aarhus, Denmark

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Abstract. In an examination of Danish children aged 0 to 4 years, with congenital sensorineural hearing loss, 60% (39/65) had rubella antibody, compared with only 23% (17/72) in an age-matched control group. Based upon these results and similar investigations reported in the literature, it is concluded that serological testing for rubella antibody has proven to be of major importance in the evaluation of etiological factors of congenital hearing loss. Although diagnostic conclusions cannot be drawn on the basis of serological testing per se in the individual child, it is important to estimate the incidence of fetal rubella infection as the cause of congenital hearing loss, since this type of hearing loss may be prevented by active immunization.

The most common manifestation of fetal rubella infection is a congenital sensorineural hearing loss, which consistently occurs alone. Due to this and to the high incidence of subclinical rubella infection during pregnancy, it has been suggested that the number of children with "rubella deafness" has been underestimated (Karmody, 1968; Gumpel et al., 1971).

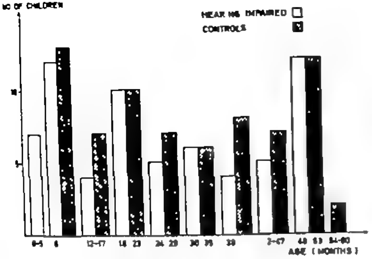
Recognition of fetal rubella infection as the cause of congenital hearing loss is of major clinical importance, both to the parents, who can be assured that rubella deafness is unlikely to occur in subsequent children, and to the child, who needs follow-up to detect any further deterioration of its ability to hear. Since congenital hearing impairment due to fetal rubella infection can be prevented by active immunization, it is of major practical importance to determine the frequency of this cause of hearing loss when considering the introduction of active immunization (Peckham et al., 1979).

A serodiagnosis of congenital rubella may be established by demonstration of rubella IgM antibodies in serum samples from infants until the age of 6–12 months (Alford, 1965; Baublis & Brown, 1968). Alternatively, the diagnosis may be based upon the persistence of rubella antibodies in samples drawn after the age of 6–12 months, as the passively transferred maternal IgG antibodies have usually disappeared by the age. However, as rubella antibody due to postnatal infection is found in approximately 5–10% of children aged 0 to 4 years, the detection of rubella antibody in the individual child cannot be conclusive (Dudgeon, 1970; Gumpel et al., 1971; Peckham et al., 1979), but may be used to estimate a retrospective diagnosis (Ojala et al., 1973).

In this study, serological testing for rubella antibody was performed in a group of Danish children with congenital hearing impairment. The results are compared with an age-matched control group in order to estimate the frequency of fetal rubella infection as the cause of hearing impairment.

MATERIAL

Sera from 137 children were tested. The children were separated into three groups: (1) 7 children with an age of less than 6 months. The median age at the serological examination of this group was 2 months (range 0–5), and (2) 58 children with a median age of 29 months (range 6–52). The children in these two groups had been referred for audiological examination at State Hearing Centres and Hearing Clinics



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They were all suffering from congenital hearing loss. As a control group (3) served 72 children with a median age of 29 months (range 6-60) without clinical symptoms of hearing loss. The majority of the latter group had been admitted for adenotonsillectomy but no routine audiometric examination was performed. Controls to group (1) were not included according to the serological method applied (see below)

METHODS

In groups (1) and (2) the congenital sensorineural hearing loss was demonstrated by various audiological procedures according to the age of the patients and their ability to co-operate in general audiometric procedures. When reliable psychoacoustic thresholds were unobtainable elevated stapedial reflex thresholds elicited by contralateral stimulation by means of a Madsen impedance audiometer ZO 70 or 72, were used to estimate the hearing ability. Alternatively the hearing loss was demonstrated by cortical evoked response audiometry and/or electrocochleography (Salomon, 1977 Elberling, 1977)

Serum was collected from 5 patients in group (1) and tested for rubella IgM antibodies after separation of the immunoglobulins by rate zonal ultracentrifugation (Vesikari & Vaheri 1968 Vejtorp & Mansa 1979) Capillary blood from the remaining patients was collected on filter paper and tested for rubella haemagglutination-inhibition (HI) antibodies as previously described (Leerhøy 1968) Children with rubella HI antibody titres of 1 10 or greater were regarded as seropositive and those with titres of less than 1 10 as seronegative

Details of the maternal and family history birth weight condition of the child at birth and the presence of other defects were obtained from the clinical records on all the patients with hearing loss. Furthermore when possible information about postnatal rubella infection was included in the clinical records

The results were analysed by the Mann-Whitney U-test and the χ-square test. The level of significance was set at 0 05

RESULTS

In group (1) the serum samples from 5 patients with hearing loss contained rubella IgM antibodies. Capillary blood collected from 1 patient at the age of 5 and 12 months contained HI antibodies without any significant difference of the titres in the samples whereas an-

Table I *The number of children with additional symptoms and the degree of hearing loss in the seropositive and seronegative groups*

Below the results of the information concerning postnatal rubella infection

	Sero-positive	Sero-negative
Clinical records		
Maternal rash	11	2
Neonatal disease	4	0
Eye defects	7	1
Heart disease	4	0
Mental retardation	2	7
Complicated delivery	9	6
Family history of deafness	6	5
Degree of hearing loss		
(1) 20–50 dB HL	4	5
(2) 50–80 dB HL	12	10
(3) >80 dB HL	4	10

Postnatal rubella

	Hearing impaired			Controls		
	Yes= 10	No= 41	?= 14	Yes= 7	No= 42	?= 23
Seropositive	8	4	7	4	5	7
Seronegative	2	17	7	3	37	16

tibodies were not detected in the sample collected from 1 patient at an age of 4 months

In group (2) 34 of the 58 patients (58 %) were seropositive while only 17 out of 72 patients (23 %) in the control group (3) had rubella HI antibodies in the capillary blood The difference between the frequency of antibodies in groups (2) and (3) was statistically significant ($p<0\ 001$) In 11 seropositive hearing impaired patients additional symptoms of a congenital rubella syndrome were observed 4 patients suffered from congenital heart disease 7 from eye abnormalities and 4 had a history of neonatal disease (thrombocytopenia purpura hepatomegaly splenomegaly enlargement of lymph nodes) In 11 patients the mothers had a history of rash during pregnancy When compared with the seronegative hearing-impaired group the seropositive group exhibits more rubella symptoms than the former (see Table I) However due to the limited size of the material and the retrospective nature of the clinical records no conclusion as to the significance of additional symptoms can be drawn regarding the seropositive and seronegative hearing-impaired patients

In the seropositive group the majority of children (24) had a severe sensorineural hearing loss exceeding 80 dB HL (mean of 500, 1 000 and 2 000 Hz for the better hearing ear). Only 4 patients suffered from hearing losses of 20–50 dB HL while 12 had hearing losses of 50–80 dB HL. When compared with the seronegative group no significant difference in the degree of hearing loss was found (see Table I)

Information concerning postnatal rubella infection was obtained from 100 patients Discrepancies between the clinical information and the results of the serological testing were found However in the control group antibodies were detected in sera from only 5 of those 42 who denied postnatal rubella while antibodies were present in 24 out of 41 children with hearing impairment and no history of postnatal rubella (see Table I)

DISCUSSION

Our results are in agreement with previous investigations Thus in children aged 6 months to 5 years Ojala et al (1973) found 43 % (57/128) of hearing-impaired children being seropositive compared with only 13 % in a control group Karmody (1968) found in 30 idiopathic deaf children 74 % seropositive and only 30 % seropositive in a control group. Peckham et al (1979) found in 349 children with congenital hearing loss 24 % being seropositive in contrast to only 9 % in a control group in which hearing impairment had been excluded They also found striking differences in family history history of maternal rubella presence of other defects and adverse perinatal events In these mate— — — — — as in the

present, the ratio between the frequency of rubella antibodies in the hearing impaired group and the control group is approximately 7.3:1. However, Gumpel et al. (1971) found that 54% (61/112) of infants aged under 4 years and having congenital sensorineural hearing loss had rubella antibody, compared with 7.1% in randomly selected children of the same age, resulting in a ratio of nearly 8:1. This discrepancy may partly be explained by the fact that initially there was a bias towards serological testing of children whose mothers had had rubella during pregnancy. Furthermore, the difference in the ratios may be explained by epidemic variations in the incidence of rubella.

The great number of hearing-impaired seropositive children compared with a control group consequently means that children between 0 and 4 years who are seropositive and in whom postnatal rubella infection can be excluded ought to be referred for hearing screening procedures, as these children may be regarded as "at risk" children.

Based upon our own and previous investigations we may conclude that serological testing for rubella antibody is of major importance when evaluating the etiology of congenital sensorineural hearing impairment. The results also show that subclinical rubella infection during pregnancy is an important factor in causing congenital deafness (in our study only 11 mothers had a history of rash during pregnancy). Although our results are highly significant, no positive conclusion as to the causative factor can be based upon the mere presence of rubella antibody in the individual child with congenital hearing impairment. However, in combination with other features associated with rubella embryopathy the diagnosis can be confirmed. As retinopathy is one of the most consistent manifestations of congenital rubella, a routine eye examination is advisable in any case of congenital deafness (Cooper et al. 1967, Parving & Starup 1976, Parving, 1977).

The audiometric configuration of the hearing losses in our patients cannot be described in detail due to the lack of co-operation by the children. However, in most of the children the hearing loss was very severe and thus in agreement with the hearing impairment caused by fetal rubella infection found by Barr & Lundström (1961) and by Gumpel et al. (1971). The audiometric results, however, may rather be an expression of an early diagnosis due to the severe degree of hearing loss than to a typical rubella hearing configuration (Miller et al. 1969). This is also supported by the similarity in the predominance of severe hearing loss in the seronegative group.

The prevalence of different causing factors of hearing loss in early childhood is difficult to assess, since it varies according to the criteria and methods used to identify such cases. In a retrospective study the incidence of fetal rubella infection causing congenital sensorineural hearing loss has been estimated to be 6% in non-epidemic years and 33.7% of all cases of congenital hearing loss after epidemic years (Fisch 1969). In a prospective study an incidence of 30% was found on the basis of serological testing (Taylor 1979).

A precise estimate of the incidence of rubella deafness in this country cannot be made from the present data due to the small number of children investigated, the retrospective nature of the diagnostic procedure and the fact that serological testing was the only objective examination. However, there is no reason to believe that major differences exist in the incidence of rubella deafness between various European countries when taking the variation of epidemics into account. As rubella embryopathy is numerically by far the most important prenatally acquired cause of profound childhood deafness (Fraser 1976), an active immunization programme ought to be considered. If the vaccines available are effective and given at a suitable age, the number of children with congenital hearing impairment should decline and unnecessary suffering on the part of the children and their families could be avoided.

CONCLUSION

In order to evaluate the etiological factors of congenital hearing loss serological testing for rubella antibody has proven to be of major importance in children aged 0 to 4 years However no diagnostic conclusion can be drawn on the basis of serological testing per se in the individual child Hearing screening and ophthalmological examination are advisable in seropositive cases The great number of seropositive children with congenital hearing loss emphasizes the need for considering and planning an active immunization programme in order to prevent hearing impairment caused by fetal rubella infection

ZUSAMMENFASSUNG

Es wurden dänische Kinder mit kongenitaler sensorineurologischer Schwerhörigkeit im Alter von 0–4 Jahren untersucht 60% (39/65) hatten Rubella-Antikörper im Blut, verglichen mit nur 23% (17/72) in einer gleichaltrigen Kontrollgruppe Mit Rücksicht auf diese Resultate und gleichwertige Untersuchungen beschrieben in der Literatur muß man schließen daß serologische Untersuchungen der Rubella-Antikörper sich von größter Wichtigkeit gezeigt haben um die etiologischen Fakten der kongenitalen Schwerhörigkeit zu beurteilen Nur kann keine diagnostische Beurteilung auf Grund serologischer Untersuchung per se für das einzelne Kind gemacht werden. Es ist wichtig festzustellen ob die Röteln-Infektion der Grund der Schwerhörigkeit ist so daß man präventiv mit Impfen behandeln kann

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A Parving
Audiology Clinic
Gentofte University Hospital
2900 Hellerup
Denmark

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THE HEALING PATTERN OF EXPERIMENTAL PARS FLACCIDA PERFORATIONS

Lars-Eric Stenfors Bengt Salén and Bengt Winblad

From the Departments of Otolaryngology and Pathology University of Umeå Umeå S eden

(Received October 30 1979)

Abstract The aim of this investigation was to ascertain how traumatic perforation of the pars flaccida heals and hether this could explain the way in which the retraction pocket develops. In rats, traumatic pars flaccida perforations were performed on both ears and the healing pattern was registered after various intervals. The perforations healed in 7 10 days forming in the first instance an indrawing of the pars flaccida and after 12 14 days an adhesive retraction, i.e the pars flaccida adhered to the neck of malleus The retraction pocket was filled with wax, keratin and detritus At the base of the retraction, epithelial inclusions could be found, which could explain hy these retractions were not self-cleaning.

Many factors predisposing to the development of retractions of the tympanic attic in man have been proposed A negative pressure due to tubal dysfunction (Bezold 1890 Ojala & Saxén 1951 Tumarkin 1961 Flisberg et al. 1963 Ingelstedt & Jonson 1967 Buckingham 1970) can cause a collapse of the eardrum with retraction of the pars flaccida and a subsequent withdrawal of the upper rear quadrant of the pars tensa. Furthermore genetic factors (Schwartz 1932 Diamant 1949 Nager 1977) a small cell system (Schwartz, 1932 Diamant, 1941) persistent mesenchyme with scar formation of the attic (Steurer 1929) recurrent infections in the tubo-tympanal spatium (Schwartz, 1932 Ojala & Saxén 1951 Ojala, 1953 Friedmann 1955 Fernandez & Lindsay 1960) destruction of the eardrum architecture (Sadé & Berco 1976) as well as a faulty growth rate of the drumhead epithelium (Austin 1977) have also been considered to be of importance in the development of persistent retractions

The histological structure of the pars flaccida is different from that of the tensa portion of the tympanic membrane (Lim 1968 *a* *b* 1970) Whereas the cytological healing pattern of perforations of the tensa portion has been fairly well documented in various animal model systems very little is known about the recovery potential of the pars flaccida after experimental perforations

The aim of this investigation was to study the cytological healing dynamics of para flaccida perforations in rats For this purpose light microscopy as well as scanning electron microscopy techniques were used The findings are discussed first in relation to the healing pattern of tensa perforations and secondly in relation to the development of attic retractions

MATERIAL AND METHOD

Thirty-five healthy rats of the Sprague Dawley *strain* exhibiting a relatively large and easily accessible pars flaccida were used in the study All animals showing any sign of external *otitis* or middle ear infection were excluded The animals were kept under standard laboratory conditions. The rats were anaesthetized by an intraperitoneal injection of Ketalar® (150 mg/kg bodyweight) A subtotal central perforation was made in the pars flaccida on both ears using a standard myringotomy lancet

After periods of 30 minutes 5 and 9 days 2 and 5 weeks the tympanic membranes were inspected photographed and 7 randomly

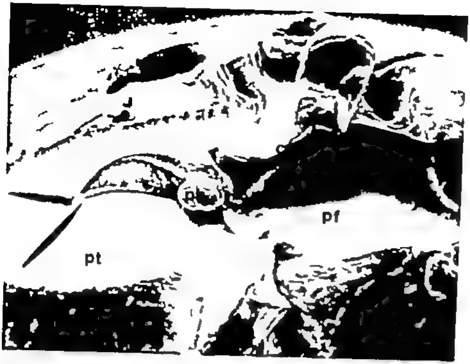

Fig 1A Scanning electron micro scopic (SEM) picture of a normal right rat tympanic membrane seen from the middle ear Pars flac cida (*pf*) is bordered by incisura Rivini and is situated lateral to the neck of the malleus (*cm*). The two fissures in the pars tensa (*pt*) leading from the umbo always developed during the critical point drying pro cedure during preparation for SEM The short process of the malleus (*pb*) is visible SEM, ×70.

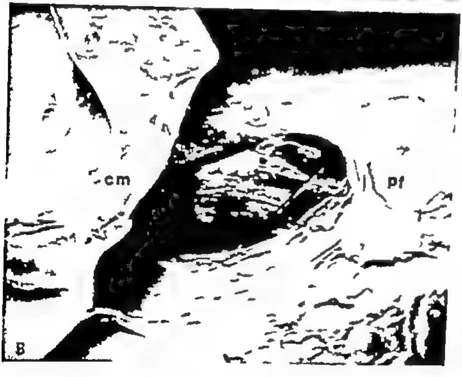

Fig 1B Pars flaccida (*pf*) half an hour after traumatic perforation. Neck of malleus (*cm*). SEM ×92.

chosen animals decapitated on each occasion The tympanic membranes with the surrounding annular ring were excised and fixed in ice cold Karnovsky s solution (paraformaldehyde/glutaraldehyde 1 1) for 24 hours From each animal one eardrum was used for scanning electron microscopy (SEM) and the other for light microscopy (LM) studies The specimens for LM were decalcified for 24 hours in New Decalk® (Histolab Betlehem Trading Gothenburg Sweden) and embedded in paraffin Sections were stained with haematoxylin-eosin and with the van Gieson stain

For SEM the specimens were dehydrated in increasing concentrations of ethanol and ethanol-amylacetate up to a concentration of 100% amylacetate and dried with the critical point drying procedure using CO_2 The specimens were then mounted on blocks and under continuous rotation and tilting covered with

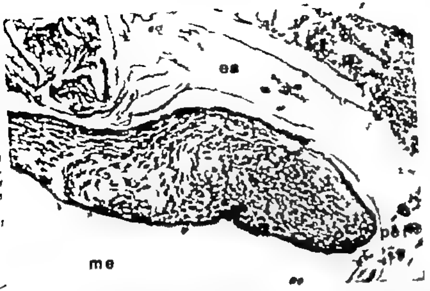

Fig. 2. Light macroscopic (LM) picture 5 days after perforation, showing at the edge hyperplastic squamous epithelium on the auditory meatal side (above). The squamous epithelium is in direct contact with the mucosal layer of the tympanic membrane (below). The connective tissue stroma centrally is hyperplastic. The perforation (*per*) is filled with keratin, fibrin and granulation tissue with inflammatory cells, among them many granulocytes. External auditory canal (*ea*). Middle ear (*me*). Htx-eosin, ×300

an approximately 200 Å thick layer of gold. The specimens were inspected using a Cambridge Stereoscan S4 scanning electron microscope.

RESULTS

Inspection of the pars flaccida at *30 minutes* showed that the perforation edges were drawn towards the periphery (Fig. 1). By LM it could be observed that the edge of the perforation was retracted and swollen and covered by a somewhat folded squamous epithelium. The short process of the malleus was partly covered by remaining fragments of the retracted pars flaccida. Only scattered inflammatory cells could be detected in the stroma at this time.

After *5 days* there was a macroscopically visible, clearly reduced yet persistent perforation, generally covered by a blood clot or a crust of wax and cell debris. The perforation boundary displayed an opaque zone which appeared thickened. There were no signs of purulent infection in this region, but a yellowish transparent fluid effusion in the attic could always be observed. LM after 5 days revealed that the material filling the perforation was fibrin and inflammatory cells—principally granulocytes—as well as debris had accumulated mainly on the auditory meatal side (Fig. 2). The perforation border of the pars flaccida was markedly thickened due to hyperplasia of the squamous epithelium as well as a thickened oedematous lamina propria. By contrast, the mucosal cells on the middle ear side of pars flaccida did not show any appreciable reaction.

After *9 days* there was still a macroscopically visible wax crust covering the pars flaccida. The latter was now completely healed but lay draped over the neck of the malleus. In one specimen the pars flaccida reached as far as to the incus. The pars flaccida was not adhesively bound to the neck of the malleus. It could be easily freed from this structure by a light suctioning. In all animals the above described serous transparent fluid could be observed in the attic, but there was no sign

Fig 3 LM 9 days after perforation The pars flaccida (*pf*) has healed somewhat thickened is retracted and covers the neck (*cm*) and the short process (*pb*) of the malleus Htx-eosin ×100

whatsoever of purulent secretion LM (Fig 3) now revealed that the pars flaccida was of an even thickness with an increase in the central connective tissue compared with normal controls In one specimen the neck of the ma and the pars flaccida were connecte granulation tissue (Fig 4)

After *2 weeks* there was still wax on the flaccida, but now a manifest retraction developed with the flaccida portion lym hesively bound to the neck of the mal Thus the pars flaccida could not be freed the neck of the malleus even when the m ear pressure was raised by air insuffl through the Eustachian tube SEM and also revealed that pars flaccida was pletely adherent to the neck of the ma (Figs *5a b* 6)

After *5 weeks* an accumulation of wax c be observed on the pars flaccida of all specimens and when the wax was remo a distinct adhesive retraction was visible one specimen (out of seven) however pars flaccida lay only around the short pro of the malleus No macroscopic changes observed in the pars tensa and no effusio the middle ear or in the attic SEM investi tion after 5 weeks showed that the pars f cida was attached tent-like to the neck of malleus (Fig 7) LM at this time also reve that the pars flaccida was adhesively boun the neck In addition at the bottom of most retracted parts there were small fo

Fig 4 In one specimen 9 days af perforation the pars flaccida (*pf*) adhering to the neck of the malle (*cm*) by a granulation-rich tissue Inflammatory cells can be observ in the attic (*at*) External auditory canal (*ea*) Middle ear (*m*). Htx eosin ×100

g. 5. SEM of (A) normal pars flaccida (pf) viewed from the medial side. (B) pars flaccida, 2 weeks after perforation, is retracted and adherent to the neck of the malleus (cm). Incus (in). SEM × 37

ith epithelial inclusions. These inclusions located on the auditory meatal side were partly filled with keratin (Fig. 8).

DISCUSSION

The flaccida portion of the tympanic membrane has a different structure and exhibits different physical properties compared with the tensa portion of the membrane. Furthermore it seems unlikely that the pars flaccida takes any part whatsoever in acoustic transmission.

It has been stated that a perforation in the pars flaccida sometimes occurs in connection with influenza (Lindsay 1934). Ruedi (1948) described flaccida perforations in patients with

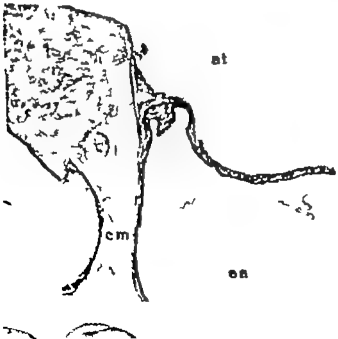

Fig. 6. LM weeks after perforation. The pars flaccida (pf) is completely adherent to the neck of the malleus (cm). The squamous epithelium and lamina propria are thin and smooth. Attic (at). External auditory canal (ea). Htx-eosin × 100.

Fig 7 SEM 5 weeks after perfo tion Pars flaccida (*pf*) is adhesi bound to the neck of the malleus (*cm*) Incus (*in*). SEM × 4

acute otitis media. Our previous studies have shown that the pars flaccida in rat perforates point like when the pressure in the middle ear reaches a certain level (Stenfors et al 1979) thus the possibility exists that a flaccida perforation could arise due to rather common place circumstances

The present investigation showed that subtotally perforated pars flaccida heals within 7–9 days covering the neck of the malleus primarily as a non adhesive indrawing which can be easily loosened This is to use Schuknecht s (1974) term a "mobile retraction. After 14 days however this retraction has

Fig 8 LM 5 weeks after perforation showing the base of a retraction pocket of the attic with epithelial inclusions filled with keratin. Neck of malleus (*cm*) External auditory canal (*ea*) Attic (*at*) Htx eosin ×300

attached firmly to the neck of the malleus and cannot be released without destroying the pars flaccida. This fixed retraction represents one of the most important pathological conditions of the middle ear (Tumarkin 1961).

It can be assumed that the epithelial inclusions in the pars flaccida, visualized by LM on the auditory meatal side after 2 and 3 weeks in which keratin and detritus accumulate help to prevent the retraction pockets from spontaneous cleaning. Furthermore there were no signs of micro-inclusions in the middle ear nor any sign of bone erosion.

In previous studies (Reijnen & Kuijpers 1971, Carlsöö et al. 1978) it was demonstrated that tensa perforations heal initially by bridging of the defect with hyperplastic squamous epithelium supported by granulation tissue. It is evident from the findings in the present study that the initial healing dynamics of the pars flaccida follow the same pattern. Lim (1968a, b, 1970) reported that the lamina propria in the pars flaccida—both in man and in the cat—is thicker and looser than in the pars tensa. This is also the case in the rat where the pars flaccida is extremely mobile and floppy. The different physical properties as well as the different substructure of the two portions of the tympanic membrane might well explain why a perforated pars flaccida does not heal in its original plane as is the case with the tensa portion. In addition it seems possible that the accumulation of wax and detritus helps to depress the perforation borders so as to cover the neck of the malleus. Macroscopically there was no sign of any purulent secretion in the attic at any stage. After a few days however a clear yellow effusion appeared in this region. A similar transparent effusion in the attic was observed when a polyethylene grommet was installed or a perforation was performed in the pars tensa, distant from the attic (Stenfors & Winblad 1979). One might speculate that this effusion serves to protect the ossicular chain as long as the perforation is still open. The effusion disappeared soon after the perforation was healed. As mentioned above there was thus macroscopically no sign of purulent infection in the attic. However other investigations (Schwartz, 1937, Ojala & Saxén 1951, Ojala, 1953, Friedmann 1955) have shown that inflammation is an initiating and important factor in the development of retractions in the tympanic attic. It can be clearly substantiated from the present findings that attic retraction pockets in the rat can occur after a traumatic perforation without any predisposing infection.

To sum up a retraction pocket may remain unchanged as long as the outer epithelial layers migrate outwards and the retraction pocket is kept free of keratin. Should keratin accumulate within that pocket however a cholesteatoma may develop gradually increasing in size as the surface desquamation accumulates. Thus the adhesive retraction pockets filled with wax and keratin found in the present investigation may according to Nager (1977) fulfil the criterion of a "dry" or inactive cholesteatoma.

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ZUSAMMENFASSUNG

Das Ziel der Untersuchung war festzustellen wie eine Perforation der Pars flaccida heilt und ob die Entstehung von Retraktionen damit erklärt werden könnte. Bei Ratten wurde eine traumatische Perforation der Pars flaccida beider Ohren gesetzt und der Heilungsverlauf nach verschiedenen Intervallen untersucht. Die Perforationen heilten in 7–10 Tagen anfangs eine Einsenkung der Pars flaccida bildend und nach 12 14 Tagen eine adhesive Retraktion aufweisend, d.h., die Pars flaccida war mit dem Hammerhals verwachsen. Die Einsenkung war mit Wachs Keratin und Detritus ausgefüllt. Am Boden der Einsenkung konnten Plattenepitheleinschlüsse gefunden werden welches erklären könnte, warum diese Retraktionen nicht selbstreinigend sind.

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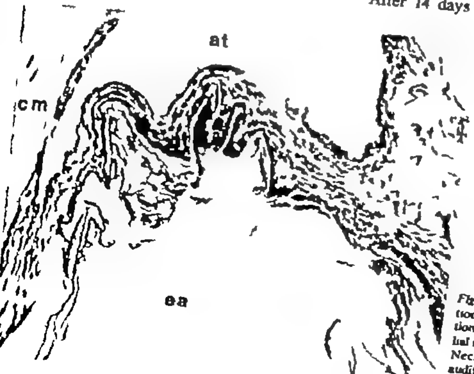

Fig 8 LM 5 weeks after perforation showing the base of a retraction pocket of the attic with epithelial inclusions filled with keratin Neck of malleus (cm) External auditory canal (ea) Attic (at) Hx eosin ×300

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ELECTROPHORETIC PROTEIN PATTERN OF HUMAN PAROTID SALIVA IN SJÖGREN'S SYNDROME AND SIALOSIS

Erika Benedek-Spät, Márta Puskás and Judit Rajki

From the Department of Oral Surgery and First Institute of Chemistry and Biochemistry, Semmelweis University Medical School, Budapest, Hungary

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Abstract. The electrophoretic pattern of the basic proteins in parotid saliva was examined in 12 control subjects and 45 patients suffering from xerostomia and parotid swelling of diverse origin. The protein pattern in the control subjects did not differ from that observed in sialotic patients. Characteristic quantitative and qualitative alterations in protein composition were observed in Sjögren's syndrome. The concentration of the protein fractions with a low R_f value showed a considerable increase, while one or two additional protein bands with high R_f value could be detected in these patients. The appearance of these additional protein bands may prove to be a valuable laboratory indicator in the diagnostic work. If they really indicate the beginning of an autoimmune process, the disease could be diagnosed much earlier than by the methods available today.

Human parotid saliva is known to contain a large number of protein components. Salivary proteins have been studied by means of a large variety of electrophoretic techniques. Alterations in the protein composition of parotid saliva have been observed in pregnancy, diabetes, sarcoidosis as well as in inflammatory diseases of the glands (Finestone et al., 1973; Beeley & Chisholm, 1976; Smith et al., 1976; Eichner et al., 1976).

An increase in the concentration of albumin and of the proteins migrating towards the anode has been found in the parotid saliva of patients with Sjögren's syndrome (Fischer et al., 1968). Moreover, new protein bands of low isoelectric point have also been detected (Chisholm et al., 1973). However, these important observations have not yet been applied to routine clinical work. The introduction of the analysis of salivary protein composition as a diagnostic tool has been delayed by the lack of simple methods and comparable results in the literature.

Examination of the parotid flow rate and parotid salivary composition in the resting state and after stimulation may provide valuable help in distinguishing pathological processes of the parotid gland (Benedek-Spät et al., 1975; Benedek-Spät, 1978). Sialochemical studies may be especially profitable in the differential diagnosis of sialosis and inflammatory diseases of the salivary glands, especially the autoimmune sialoadenitis in Sjögren's syndrome.

As an extension of these sialochemical studies, we have examined the protein composition of the parotid saliva of healthy subjects and patients with Sjögren's syndrome or sialosis. The polyacrylamide gel electrophoresis, applied in our examinations, has been a simple and rapid method, requiring only a minute amount of saliva for the analysis. Preparation (lyophilization or concentrating) of the samples is not needed, thus the risk of changes in the protein fractions is also reduced.

MATERIAL AND METHODS

The examinations were carried out in 12 healthy subjects and 45 patients complaining of xerostomia and swelling of the parotid gland (Table I). Routine laboratory examinations and the gamma-latex test were comp-

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L. E Stenfors M.D
Dept of Otolaryngology
University of Umeå
S-901 87 Umeå
Sweden

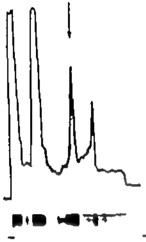


Fig. 2. Electrophoretic pattern of salivary proteins in a case of Sjögren's syndrome. The arrow indicates an additional protein band.

Fig. 3. Electrophoretic pattern of salivary proteins in case of Sjögren's syndrome. The two additional protein bands are indicated by arrows.

group of Sjögren's syndrome and are designated xerostomia group (Table I).

Saliva was collected in the fasting state between 8.00 and 9.00 a.m. by means of a thin polyethylene tube introduced into the parotid duct. Details of the technique were described previously (Benedek Spät 1973). Unstimulated saliva was collected for 10 min. After an interval of a few minutes the secretion was stimulated by a 2% solution of citric acid, of which two drops were applied every half minute on the upper surface of the tongue. Stimulated saliva was collected for 3–5 min, depending on the flow rate. The secretion rate both before and after stimulation was measured and the total protein content of the stimulated saliva was determined by the method of Lowry et al. (1951) using human serum albumin (fraction V) as a standard.

Stimulated parotid saliva was analysed for protein composition by means of electrophoresis. Saliva from the resting state was analysed for protein composition in two cases of Sjögren's syndrome. Samples of saliva (50–150 μl depending on the total protein content of the saliva) were run in a 7.5% polyacrylamide gel in 5 mM Tris-glycine buffer (pH 8.3) at 4°C. Bromphenol blue was used as marker. An initial current of 0.5 mA/tube was applied for 30 min, followed by 4 mA/tube. The gels were stained with a 0.1% solution of amido black in 7% acetic acid for 30 min and were rinsed afterwards several times with 7% acetic acid. The electrophoretograms were evaluated quantitatively by means of a Chromoscan

Fig. 4. Characteristic electrophotograms in the following order (from top to bottom): 1, 2, control; 3–6, Sjögren's syndrome with one or two additional protein bands; 7, 8, sialosis.

Table I *Average ages resting and stimulated parotid flow rates and protein concentrations of stimulated parotid saliva in controls and the different groups of patients*

	Number of patients	Age	Resting flow (ml/min)	Stimulated Flow (ml/min)	Stimulated Protein (mg/100 ml)
Control	12	30 (21–54)	0 035±0 007	0.53±0 12	240±40
Sialosis	12	52 (35–63)	0 063±0 016	0.59±0 12	263±56
Sjögren s syndrome	28*	52 (26–78)	0 017±0 004	0 19±0 03	369±30
Xerostomia	5	61 (52–76)	0 036±0 009	0 19±0 05	388±41

Unstimulated saliva is unmeasurably low in 11 cases.

leted with sialography of at least one parotid in each patient

The group of sialosis consisted of twelve patients The term sialosis in used to designate the painless non neoplastic non-inflammatory enlargement of the salivary glands (Rauch & Gorlin 1970) The sialotic patients in this study had bilateral enlargement of the parotid gland The sialographic examination showed a normal salivary duct configuration in each of the patients the result of the gamma latex test being negative Cultures of the parotid saliva were sterile the sialochemical examination revealed low sodium and protein concentrations and increased potassium secretion rates characteristic of sialotic patients (Benedek-Spät 1978) The parotid swelling was often associated with endocrine disturbances (chiefly diabetes mellitus or dysmenorrhoea) hypertension chronic alcoholism and neurotic complaints

Diagnosis of Sjögren s syndrome has been established in 28 cases Patients were included in this study when at least two of three major components of Sjögren s syndrome (xerostomia kerato-conjunctivitis sicca rheumatoid arthritis or some other collagen tissue disorder) could be demonstrated Patients with xerostomia and kerato-conjunctivitis sicca only were referred to as having sicca syndrome The characteristic signs of kerato-conjunctivitis sicca were demonstrated by the Schirmer test and by rose bengal or fluorescein staining of the conjunctiva and cornea.

The diagnosis of definite rheumatoid arthritis was based on the criteria of the American Rheumatism Association (Ropes et al 1959). A more detailed presentation of the diagnostic data is given in Table II

Xerostomia and recurrent swelling of the parotids were the only pathological signs in 5 cases For lack of further clinical symptoms these patients have not been included in the

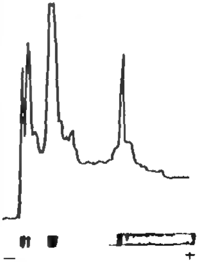
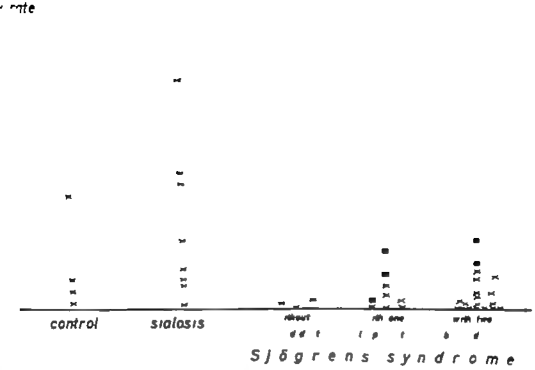


Fig 1 Electrophoretic pattern of salivary proteins in a healthy subject.



Individual parotid flow rates in control subjects he different groups of patients. ■ patients with xerostomia and swelling of the salivary glands without further characteristic signs of Sjögren syndrome

sily distinguished A similar electro- ic pattern including the presence of one additional protein bands, was found in liva of the 5 patients who had xero- a and recurrent parotid swellings but no symptoms

typical protein pattern of the examined s is shown in Fig. 4 the *R* values of the dual bands are summarized in Fig 5

Table I sets out the ages of patients as well as unstimulated and stimulated flow rates and protein concentrations of the saliva in the dif ferent groups of patients The mean protein concentration in the saliva of patients with Sjögren s syndrome or xerostomia alone was moderately higher than that in control or sialotic saliva. Distribution of the patients with Sjögren s syndrome according to the electro-

III *Distribution of the patients with Sjögre s syndrome according to the electrophoretic rn of parotid salivary proteins*

	Number of patients	Age	Flow ml/min		Protein (mg/100 ml)
			Unstimulated	Stimulated	
ut additional band	3	48 (43–53)	0 006	0.10±0 [illegible]	233±23
one additional band	6	52 (26–78)	0 013±0 004	0.20±0 04	382±58
two additional bands	19	53 (32–64)	0 020±0 006	0.20±0 04	390±38

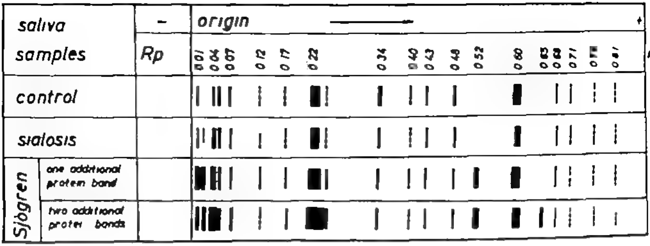


Fig 5 Characteristic *R* values in saliva samples of different origins

(Joyce Loebl) densitometer The protein bands were characterized by the R_p values i e the ratio of the migration of the protein fraction and the indicator dye Electrophoretic analyses were repeated after a one year interval in 5 cases of Sjögren s syndrome

RESULTS

A typical electrophoretic separation of the proteins in the parotid saliva of healthy control subjects is shown in Fig 1 Individual variations were observed in respect of the densitometric intensity of the separated bands and in the existence of some minor bands

Nevertheless the distribution of protein fractions and the R_p values of the major fractions were uniform in the control group The electrophoretic pattern of the saliva of sialotic patients did not differ from that of the control group

The electrophoretic pattern of the parotid saliva was examined in 28 patients with Sjögren s syndrome With the exception of 3 cases the pattern differed significantly from the control In general the concentration of the fractions with low R_p values (0 01–0 07) often increased to the extent that the individual bands could not be separated The intensity of the fraction with an R_p value of 0.22 also increased Beside these quantitative changes new protein bands could also be observed One additional band ($R = 0.52$) was detected in 6 cases two additional bands ($R_p =$ 0.52 and 0 65 resp) were found in 19 cases (Figs 2 and 3) The fraction with an R_p of 0 52 could be clearly distinguished in each case and gave an intense staining The other additional band ($R_p = 0$ 65) was distinctly separated in 15 cases and vaguely recognized in 4 other cases In 2 of these 4 cases we could collect unstimulated saliva in sufficient quantities for electrophoretic analysis In these samples both additional protein bands could

Table II *Clinical data of the patients with Sjögren s syndrome*

	Number of patients	Sialectasis	Salivary gland swelling
Sicca syndrome (xerostomia and keratoconjunctivitis sicca)	8	3	4
Sicca syndrome and definite rheumatoid arthritis	13	5	8
Sicca syndrome and systemic lupus erythematodes	2	1	1
Sicca syndrome and scleroderma	1	–	–
Sicca syndrome and dermatomyositis	1	–	–
Rheumatoid arthritis with xerostomia or keratoconjunctivitis sicca	3	1	1

occasional lack of some minor fractions in the electrophoretic pattern of control parotid saliva. No difference was observed between the protein patterns of control and sialotic subjects.

The electrophoretic pattern in Sjögren s syndrome exhibited characteristic differences as compared with the control in 25 of 28 examined patients. There was an increase in the amount of protein fractions with the lowest R_p value and with an R of 0 22 One or two additional protein bands could also be discerned The alterations in the composition of salivary proteins in Sjögren s syndrome was first reported by Fischer et al (1968) and Chisholm et al (1973) Fischer observed a correlation between the severity of clinical symptoms and the concentration of those proteins migrating the most rapidly towards the anode

Additional protein bands with a low isoelectric point were detected by Chisholm (1973) in the saliva of patients with Sjögren s syndrome These bands could also be observed in cases of rheumatoid arthritis though they were usually absent in sicca syndrome

Our examinations failed to disclose any unambiguous correlation between the progress of the pathological process and the appearance of extra protein bands The individual values of saliva flow rate indicate that changes in the electrophoretic pattern cannot be attributed to a diminution of flow rate alone (cf Fig 6) Equally low flow rates are associated with unaltered protein pattern in control and sialotic subjects but with characteristic changes in the protein pattern in Sjogren s syndrome. Some explanation is required for our other observation that in the three cases of Sjögren s syndrome where we found normal protein composition very low flow rate values were not associated with the characteristically high protein concentration (Table III)

Increased concentrations of immunoglobulins and albumin in the saliva were observed in Sjögren s syndrome although this increase was smaller than that found in other inflammatory processes (Fischer et al 1968 Bluestone et al 1972 Mandel & Maurmash 1973 1976 Eichner et al. 1976) Based on the results of Steiner et al. (1968) Bellavia (1971) and Eichner et al (1976) it may be assumed that in our samples the intensively stained protein fractions of low R values were immunoglobulins, while the $R = 0.22$ fraction also well stained may contain amylase isozymes Our examinations do not give any information on the chemical character and biological role of the additional protein fractions It seems probable that the additional $R_p = 0.52$ and $R = 0.65$ bands are identical with the anodal proteins of low isoelectric point, respectively described by Chisholm et al (1973)

The appearance of additional protein bands may prove a valuable laboratory indicator in diagnostic work Their specificity for Sjögren s syndrome requires further examination Provided they indicate the genesis of an autoimmune process the disease may be diagnosed much earlier than by means of the methods available today

ZUSAMMENFASSUNG

Die elektrophoretische Probe der basischen Eiweiße vom Parotisspeichel wurde bei 12 Kontrollpersonen und 45 Patienten, die an Xerostomie verschiedenen Ursprungs litten, verglichen. Die elektrophoretische Analyse der Speicheleiweiße ist in Kontrollpersonen und in sialotischen Patienten praktisch identisch. Gleichzeitig sind charakteristische quantitative und qualitative Abweichungen in dem Sjögren-Syndrom zu beobachten Die Konzentration der Proteinfraktionen mit niedrigem Rp-Wert zeigt eine bedeutende Erhöhung, während bei diesen Patienten ein oder zwei zusätzliche Proteinstreifen mit hohem Rp-Wert nachweisbar war Das Erscheinen der zusätzlichen Proteinstreifen kann eine wertvolle diagnostische Hilfe sein. Wenn dieses Erscheinen auf die Bildung eines autoimmunen Prozesses charakteristisch ist, kann die Krankheit eventuell schon früher diagnostiziert werden als mit Hilfe der bis jetzt zu Verfügung stehenden übrigen diagnostischen Mittel

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Table IV *Relation of clinical symptoms and electrophoretic pattern of parotid salivary proteins in Sjögren s syndrome*

Electrophoretic pattern	*n*	Sicca syndrome	Sicca syndrome and connective tissue disorder	Rheumatoid arthritis and xerostomia or keratoconj. sicca
Without additional protein band	3	1	1	1
With one additional protein band	6	2	4	–
With two additional protein bands	19	5	12	2

phoretic pattern is shown in Table III Attention is called to the fact that in the three cases where the electrophoretic pattern did not differ from the control the significantly reduced flow rate was associated with normal concentration of proteins

Individual flow rates in the various groups with special respect to the appearance of additional protein bands are shown in Fig 6

We could not detect any unambiguous correlation between the presence of additional protein bands and the various clinical symptoms (Table IV) The relationship between the electrophoretic pattern and some laboratory data is shown in Table V The appearance of additional protein bands was associated with swelling of the salivary glands in half of the cases while sialographic alterations and/or the rheumatoid factor could be detected in about one third of the cases

Electrophoretic examinations were repeated one year later in 5 patients with Sjögren s syndrome An unaltered electrophoretic pattern was found in 3 subjects In one subject originally exhibiting one extra band only worsening of the clinical symptoms was associated with the distinct appearance of the other extra band too On the other hand in a case of significant amelioration of the state of the patient both additional bands had disappeared during the year

DISCUSSION

Electrophoretic examinations of the saliva have generally been hampered by the low and heterogeneous protein content of the saliva and the dependence of protein migration on the concentration of inorganic ions Concentration or dialysis of saliva samples regularly carried out prior to electrophoresis may result in the transformation of some proteins. Our method avoids these steps and thus provides more reliable information on the distribution of protein fractions in the saliva. The resolution of these fractions is comparable to that reported by other authors (Steiner & Keller 1968 Beeley 1969 Bellavia 1971) using more complicated techniques The present method has a high sensitivity inasmuch as the analysis can also be carried out with minute volumes (50–150 μl) of saliva. This advantage provides an opportunity for protein analysis even in cases of significantly reduced flow rate

Minor differences were observed in the intensity of the individual bands as also was the

Table V *Relation of some laboratory data and the electrophoretic pattern of parotid salivary proteins in Sjögren s syndrome*

Electrophoretic pattern		Gamma-late test positive	Sialectasis	Swelling of salivary glands
Without additional protein band	3	1	1	1
With one additional protein band	6	1	–	1
With two additional protein bands	19	9	9	1

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IDENTIFICATION OF MIDDLE EAR DISEASE

Ulf Renvall Jan Jarlstedt and Jörgen Holmquist

From the E.N.T. Dept. Sahlgren Hospital and Östra Sjukhuset Göteborg University of Göteborg Göteborg S[illegible]de

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Abstract Since impedance measurements were introduced as mean of identifying ears affected by middle ear disease, efforts have been concentrated on the identifying of ears in which effusion is present. The shape of the tympanogram has been analysed and it has been proposed that ears producing a shallow tympanometric pattern are more likely to contain fluid in the middle ear than are ears having tympanogram with large peak. In this communication the great individual variation of tympanograms is shown and it is suggested that two categories of tympanogram should be considered namely 1) flat tympanogram, and 2) tympanograms in which the middle ear pressure can be determined.

In recent years there has been much interest in identifying fluid in the middle ear. Impedance measurements have been employed in order to determine the presence of middle ear effusion (Berry et al. 1975 Brooks 1969 Renvall et al. 1973 Renvall et al. 1975). Also the combining of different test methods such as impedance tests pure tone test and otologic evaluation has been proposed with the aim of increasing confidence in the diagnosis of middle ear effusion (Grimaldi, 1975). This interest in revealing effusion in the middle ear has rather masked the fact that it is as important to identify ears with high negative middle ear air pressure since these ears are also capable of developing retractions, adhesions chronic otitis media and cholesteatoma (Gundersen & Tonning 1976 Smyth 1976 Tos & Poulsen 1976). In the present paper data are presented which indicate that the recording of middle ear air pressure is the best method for diagnosis of ears affected by middle ear disease or ears with the potential to develop middle ear disease.

MATERIAL AND PROCEDURE

The study comprises 103 children ranging in age from 4 to 10 years. All had been recommended for myringotomy on the basis of otologic indications of middle ear effusion. Preoperative tympanometry was performed in all ears 30 to 60 minutes before myringotomy. General anesthesia was used for all myringotomies. At surgery the presence of absence and type of effusion in the middle ear was noted. When indicated ventilating tubes were inserted. A Madsen electroacoustic impedance bridge 625 HZ (Model ZO-70) and an x-y plotter were used for the tympanogram recording. Tympanograms were automatically plotted between ± 200 mmH_2O. If no impedance minimum was found within this pressure range the pressure was manually reduced to -600 mmH_2O in order to identify the impedance minimum. The tympanogram were

Table I *Findings at surgery in ears with flat tympanograms*

Type of effusion	Number of ears
Serous	54
Mucoid	[illegible]
No effusion	4
Total	77

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Dr Erika Benedek Spät
Dept of Oral Surgery
Semmelweiss Univ Medical School
Budapest
Hungary

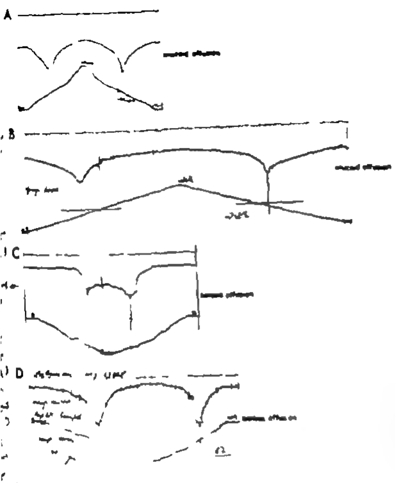

Fig. 3. Tympanograms from 4 different patients associated with mucoid respectively serous effusion in the middle ear.

in Fig. 5. In one ear (Fig. 5A) no effusion was found and in the other (Fig. 5B) serous effusion was found at myringotomy. Fig. 6 illustrates tympanograms from two otomicroscopically normal ears. In one ear (Fig. 6A) the tympanogram has a deep peak and in the other (Fig. 6B) the tympanogram has a shallow peak. A comparison was made between different levels of the middle ear pressure observed and the presence or absence of effusion. The pressure distribution is shown in Table III. If middle ear pressure = −150 mmH$_2$O is used as a limit for abnormality, 11 ears with effusion would not have been classified as abnormal

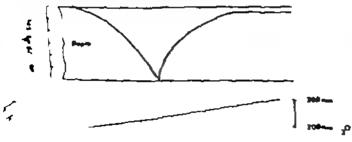

Fig. 4. Illustration of measurement of the depth of tympanogram.

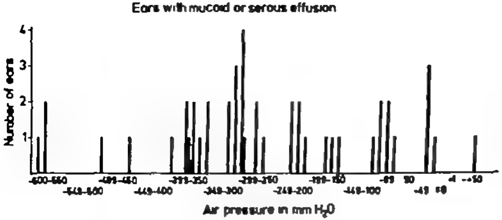

Fig 1 Ears (46) with effusion in which the middle ear pressure could be determined.

differentiated into two types an impedance minimum could (1) or could not (2) be identified

Correlation Between Type of Tympanogram and Middle Ear Effusion

Tympanograms from 158 ears were obtained In 77 ears the tympanogram was flat Table I shows that effusion was demonstrated in 73 of these ears in 4 ears no effusion was found at myringotomy Of the ears with no effusion two demonstrated adhesive processes while two appeared normal In 81 ears the middle ear pressure could be determined: effusion was demonstrated at myringotomy in 46 of these The distribution of the middle ear pressure in these ears is shown in Fig 1 Fig 2 shows the distribution of middle ear pressure in 28 ears with mucoid effusions and the pressure distribution in 18 ears with serous effusion Fig. 3 illustrates tympanograms from two ears in which mucoid effusion was found at myringotomy (Fig 3 A B) and two ears in which serous effusion was found (Fig 3 C D) Mean peak depth of the tympanograms from ears with serous effusion was 15 mm—and from ears with mucoid effusion 10 mm (Table II). The depth of tympanogram peaks was measured according to (Fig 4) Examples of tympanograms with shallow peaks are presented

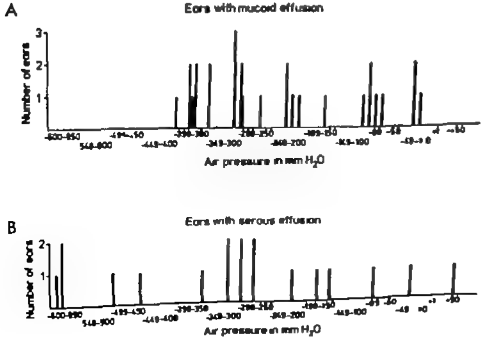

Fig 2 Surgical findings in 46 ears in which the middle ear pressure was determined 30–60 min before surgery

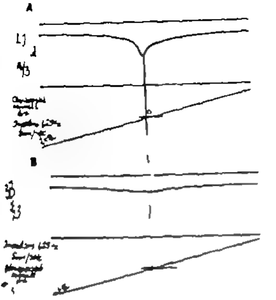

Fig. 6. Tympanograms from two otomicroscopically normal ears.

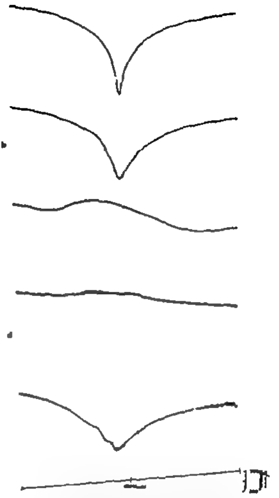

Fig. 7. Tympanometry performed with different amounts of water in the middle ear. ( ) Middle ear without water; (*b*) water-line 1– mm above umbo; ( ) water-line at umbo; (*d*) water-line reaching top of the tympanic membrane; ( ) water sucked out from the middle ear.

predicting effusion the great individual variability in the shape of the tympanogram must be taken into account. In Fig. 3 A B D it was demonstrated that ears with effusion may be

Table III. *Middle ear pressure correlated to surgical findings*

Middle ear pressure (mmH_2O)	Ears
Effusion	
> 150	11
< 150	108
No effusion	
> 150	19
150	20
Effusion	
100	8
100	111
No effusion	
100	11
< 100	25
Effusion	
200	14
200	105
No effusion	
200	19
< 200	20

associated with a tympanogram shape which cannot be differentiated from tympanograms from normal ears and in Fig. 6 the great variability of the shape of the tympanograms between otomicroscopically normal ears is shown. It is therefore suggested that only two categories of tympanogram should be considered in relation to ears with possible disease (serous mucoid otitis media), namely (1) flat tympanogram and (2) tympanograms in which the middle ear pressure can be determined. The distinction between these two classes

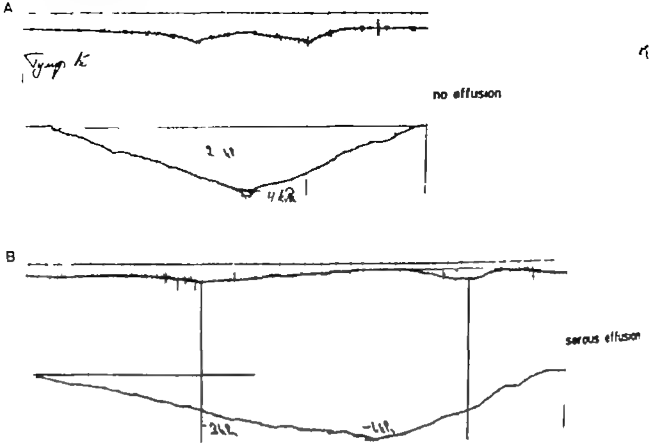

Fig 5 Tympanograms from two ears in which shallow peaks was demonstrated. In (A) no effusion was found and in (B) effusion was demonstrated at surgery

The results when $\leqslant -100$ mmH$_2$O or $\leqslant -200$ mmH$_2$O are used as a limit for abnormality are also given in Table III False negatives with effusion increased from 8 to 14 and false positives without effusion decreased from 28 to 20 with criteria of $\leqslant -100$ and $\leqslant -200$ mmH$_2$O respectively as the limit for abnormality

DISCUSSION

Since impedance measurements were introduced as a means to identify ears affected by middle ear disease efforts have been concentrated on the identifying of ears in which effusion is present The shape of the tympanogram has been analyzed and it has been proposed that ears with shallow tympanometric pattern (high impedance system) are more likely to contain fluid in the middle ear than ears having a tympanogram with a large peak (low impedance system) (Berry et al 1975 Orchich et al 1978) This is in agreement with experimental study of human temporal bones in which it is demonstrated that fluid in the middle ear influenced tympanogram shape (Renvall et al 1975) (see Fig. 7) Tympanograms become shallower as the amount of fluid in the middle ear increases If however the tympanogram is to be used as a basis for

Table II *Registered peak depth from tympanograms from patients with mucoid respectively serous effusion in the middle ear*

Number of ears	Type of effusion	Tympanogram height (mean) (mm)	Standard deviation
28	Mucoid	18.2	15 4
15	Serous	14 9	9 6
35	No effusion	4 9	10.9

relation [redacted] middle ear disease it is most important that the limit for pathologic value is realistic. If the limit selected results in too many false-negatives the usefulness of tympanometry as a diagnostic tool will be limited. As a limit for screening purposes in 7 year olds ≤ -150 mmH_2O [redacted] recommended (Renvall & Lidén, 1979). This value could also serve as a guideline in otologic practice and if used together with the case history and tympanic membrane inspection the tympanometric evaluation would be an invaluable tool for dealing with ears affected by middle ear disease.

ACKNOWLEDGEMENTS

Professor Josef M. Miller Seattle USA reviewed the manuscript and gave valuable help. Harald Nyman did the statistical analysis. This work was supported by grant from the Swedish Medical Research Council (no. B 79-17X 5034-01).

ZUSAMMENFASSUNG

Seit die Impedanz-Technik als Mittel, die Mittelohrpathologie zu identifizieren eingeführt worden ist, hat sich das große Interesse darauf konzentriert, Mittelohrergüsse festzustellen. Das Aussehen des Tympanogramms wurde analysiert, und es ist festgestellt worden, daß es mehr wahrscheinlich ist, Ergüsse in den Ohren mit flachen tympanometrischen Mustern zu finden als in den Ohren, welche eine tympanometrische Kurve mit markanter Spitze zeigten. In diesem Artikel wird die große individuelle Variation des Tympanogramms festgestellt und es ist ein Vorschlag gemacht worden, welcher nur zwei Tympanogrammarten diskutiert: 1) Tympanogramm mit gerader Kurve 2) Tympanogramm, in welchem man den Mittelohrdruck feststellen kann.

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U. Renvall, M.D.
Dept. of Otolaryngology
Östra Sjukhuset
Smörslottsgatan 1
S-416 85 Göteborg
Sweden

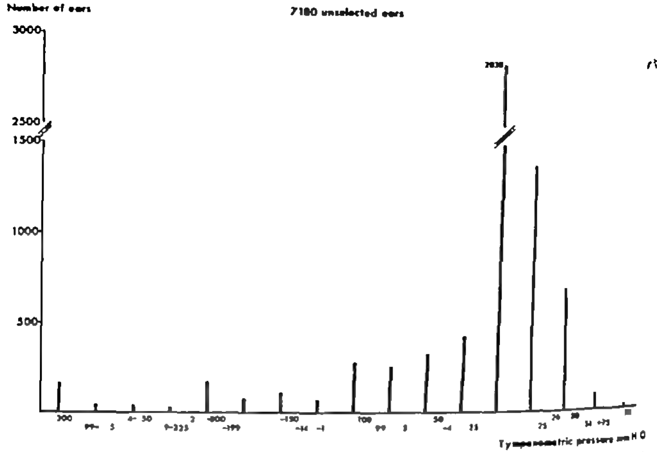

Fig 8 Non-selected ears from 7 year-olds In 10% of the ears tympanometry demonstrated middle ear pressure $\leqslant -150$ mmH_2O or a flat tympanogram. (From Renvall & Lidén 1978 Grune & Stratton, Inc)

rests in part of course upon the highest negative pressure used in the ear canal to identify the impedance minimum

From Table III it is also obvious that there are ears with considerable negative pressures ($\leqslant -200$ mmH_2O) in which no effusion could be demonstrated at myringotomy The large negative pressure in the middle ear is in itself a sign of deficient Eustachian tube function and an earlier study (Renvall et al 1978) demonstrates that in ears in which reduced middle ear pressure has been demonstrated earlier 16% still had reduced middle ear pressure 3 years later compared with 4% in non-selected group Thus even though no effusion could be observed in these ears with large negative pressures future evaluation is considered important to prevent development of retracted tympanic membranes and chronic middle ear disease

In ears in which the middle ear pressure can be determined it is necessary to decide at which pressure level the middle ear pressure shall be considered pathologic From Fig 1 it is clear that effusion can be present in ears with a middle ear pressure within ± 100 mmH_2O i e a pressure which may be considered as normal In Table III it is shown that in the ± 150 mmH_2O pressure group 11 ears demonstrated effusion at surgery while in 19 ears no effusion was found at paracentesis. It must however be noted that this study was performed on a selected group and in all ears there was otologic suspicion of effusion In a non selected material on 7 year-olds 10% had a middle ear pressure $\leqslant -150$ mmH_2O (Fig 7), in non selected 3-year-olds (Fiellau Nikolajsen et al 1977) 38 2% of the ears had a middle ear pressure of $\leqslant -100$ mmH_2O and in 3-month-olds (Poulsen & Tos 1978) 38.2% had a middle ear pressure of $\leqslant -150$ mmH_2O When we discuss the use of tympanometry in

relation to middle ear disease it is most important that the limit for pathologic value is realistic. If the limit selected results in too many false-negatives the usefulness of tympanometry as a diagnostic tool will be limited. As a limit for screening purposes in 7 year olds ≤ -150 mmH_2O is recommended (Renvall & Liden 1979). This value could also serve as a guideline in otologic practice and if used together with the case history and tympanic membrane inspection the tympanometric evaluation would be an invaluable tool for dealing with ears affected by middle ear disease.

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U. Renvall, M.D.
Dept. of Otolaryngology
Östra Sjukhuset
Smörslottsgatan 1
S-416 85 Göteborg
Sweden

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IMMUNE COMPLEXES AND COMPLEMENT IN SEROUS AND MUCOID OTITIS MEDIA

A B Laurell [1] N I Nilsson[2] and K Prellner[2]

From the Department of Medical Microbiology Section for Immunology and the [2]Department of Otorhinolaryngology University Hospital of Lund Lund Sweden

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Abstract The occurrence and quantity of immune complexes in middle ear effusion (MEE) and serum, as well as serum levels of complement (C) factors were investigated in patients with chronic otitis media. Immune complexes were demonstrated in 85% of the serous MEE and in 28% of the sera. Depressed C1q values and presence of abnormal complexes composed of subcomponents of the first C factor indicated a disturbed function of the C system Activation of C by the classical pathway was demonstrated in 23% of the patients. Decreased levels of properdin were also noted The disorders within the C system tended to normalize as the otitis subsided

The etiology of chronic serous or mucoid otitis media (OME) is still not clarified Bacterial cultures from the middle ear effusions have most often proved negative (Liu et al 1976 Giebink et al 1979) The concept that chronic serous otitis media is immunologically mediated was suggested by Veltri & Sprinkle (1976) In one study the presence of immune complexes in middle ear effusions has been reported (Maxim et al 1977)

Complement (C) is a well defined system of plasma proteins which includes several unique enzymes C is involved in the defence against invading microorganisms and in inflammation Complement is activated by immune complexes but also by other mechanisms In the classical pathway of C activation the first factors of complement (C1q C1r C1s) are bound and activated by antigen–antibody complexes If this occurs on a bacterial cell the complement cascade engages C3 which in its activated form C3b is bound to the cell surface It has been shown that pneumococci are taken up by polymorphonuclear leukocytes mainly when coated with antibody and complement and that opsonization and phagocytosis of pneumococci are essentially dependent on C3b bound to the bacterial cell (Winkelstein et al 1975)

Disorders within the complement system have been reported in children with relapsing otitis media due to pneumococci (Johnson et al 1977) Decreased C1q together with normal or elevated levels of C1r and C1s were found in 60% of these patients In addition increased amounts of complexes composed of C1 subcomponents C1r–C1s and C1̄r–C1̄s–C1̄ inactivator (C1̄r–C1̄s–C1̄ I A) were a constant finding

In order to elucidate mechanisms contributing to the chronic course in serous or mucoid otitis media, studies were undertaken concerning the occurrence and quantity of immune complexes in middle ear effusion (MEE) and serum and the pattern of some of the complement components was analysed

MATERIALS AND METHODS

Patients

Eighty-six patients (aged 1–8 years mean 3 9) with chronic otitis media with effusion (OME) serous or mucoid were investigated The duration of OME was between one and 6 months Myringotomy was performed under

Table I *Bacteriological findings in nasopharyngeal samples in patients with chronic otitis media and from healthy children*

	Number of individuals harbouring bacteria	
Bacteria	Patients (n=86)	Healthy individuals (n=29)
S. pneumoniae	35	3
H. influenzae	21	7
Gr. A streptococci	2	1
B. catarrhalis	34	22
No pathogens isolated	14	10

general anesthesia in all patients. For comparison, 29 children aged between 2 and 10 years (mean, 6.4) were also investigated. The samples were obtained under general anesthesia. The control group comprised children under surgical treatment for hernia inguinalis, phimosis, naevus, or short frenulum linguae.

Middle ear effusions were collected from 20 patients with serous OME by aspiration through the ear drum.

Serum and EDTA-plasma (Na_4EDTA 5 mmol/l) were obtained at the time of the first myringotomy and again in 31 patients 4 weeks later. Serum, plasma and middle ear effusions were frozen within 3 hours of sampling and stored in aliquots at −80°C until analysed.

Bacteriological examination

Specimens were obtained from nasopharynx using a sterile cotton swab and transported to the Bacteriology Department in haematin agar tubes within 24 hours. The nasophraryngeal specimens were inoculated on haematin agar and blood agar and the plates incubated at 37°C for 18 hours in 6% CO_2 atmosphere and anaerobically (Gas Pak® System) respectively.

Pneumococci, β-hemolytic streptococci and *H. influenzae* were identified using standard methods. Streptococci were serologically grouped by coagglutination (Christensen et al 1973). *B. catarrhalis* was identified as described by Kamme (1970).

Serum IgG was quantitated according to Grubb (1970). The normal ranges used for the different age groups were those detailed by Johansson & Berg (1967).

C-reactive protein was estimated by electroimmunoassay (Kindmark 1969).

Quantitation of complement components

Levels of C1q, C1s, C3, C4 and properdin were measured by electroimmunoassay (Laurell, C.-B. 1972, Laurell, A.-B. et al. 1978). The normal ranges referred to are those given for adults (Sjöholm 1975). Children aged between 2 and 10 have similar levels of C1q. However, C3 levels in this age group are approximately 10% higher when compared with adults (Yonemasu et al. 1978).

C1 subcomponent complexes. Complexes of C1r-C1s and C1r-C1s-C1 IA, respectively, were demonstrated by crossed immunoelectrophoresis (Laurell et al. 1976).

C1r-C1s-C1 IA complexes were quantitated immunochemically (Laurell et al. 1979). The normal range of C1r-C1s-C1 IA was 11–25% of a reference serum (Laurell et al. 1979).

C1q binding substances were examined by the C1q deviation test, C1q DT (Sobel et al. 1975) and by the C1q binding assay, C1q BA (Zubler et al. 1976). Values were expressed in equivalents of heat aggregated IgG (μg/ml). For each experiment, calibration curves were prepared by adding various amounts of aggregated IgG to fresh serum.

Statistical methods

Student's *t*-test for paired and unpaired data and the χ^2 test were used.

RESULTS

I. Comparison between patients with chronic OME and healthy children

Bacteriological findings. S. pneumoniae, H. influenzae, group A streptococci and *B. catarrhalis* were isolated from the nasopharyngeal samples in 72 of 86 (84%) of the OME patients and in 19 of 29 (66%) of the

Table II *Complement factor levels in sera from 86 patients with chronic OME and from 29 healthy children*

Complement factor	Mean value ±1 S D in		Statistical difference in mean value
	patients	healthy children	
C1q	8[illegible]±14%	86±15%	No difference ($p>0\ 05$)
C1s	112±17%	97±15%	No difference ($p>0\ 05$)
C1s–C1q	20±16	11±15	Significant difference ($p<0\ 02$)
C3	107±16%	98±21%	Significant difference ($p<0.05$)
C4	99±33%	95±30%	No difference ($p>0\ 05$)
Properdin	85±17%	105±28%	Significant difference ($p<0\ 001$)

Numerical difference between C1s and C1q levels in individual sera

control patients (Table I) Thirty-one of all samples harboured more than one pathogen

Immunoglobulin G levels were within normal ranges No increase in C reactive protein (i e less than 12 mg/l) was found

Levels of complement component in serum The mean C1q and C1s values of sera from OME patients did not differ from those of the controls ($p>0\ 05$) (Table II) However C1q was disproportionately low compared with C1s in individual sera from most of the OME patients Compared with the control group the mean values for this numerical difference between C1s and C1q was statistically significant ($p<0\ 02$ Table II)

C4 values did not differ between the two groups (Table II) C3 were within the normal ranges in all sera but the mean value was higher in the OME patients than in control sera ($p<0\ 05$)

The properdin values were lower in sera from OME patients than in sera from the control group ($p<0\ 001$ Table II)

C1 subcomponent complexes Increased amounts of C$\bar{1}$r–C1s–C$\bar{1}$ IA complexes were found in 23% of sera from OME patients The levels of C$\bar{1}$r–C$\bar{1}$s–C$\bar{1}$ IA complexes were within the normal range in all sera from the control group (Table III) This difference was statistically significant ($p<0\ 025$)

C1r–C1s complexes were detected in 41% of the OME patients and in 10% in the control group ($p<0\ 01$ Table III)

C1r–C1s complexes were found more frequently in sera where a discrepancy between C1s and C1q values was present (Fig. 1) C1r–C1s complexes were present in 33 of 72 (46%) patient sera from whom potentially pathogenic bacteria were isolated and in 3 of 14 (21%) sera from patients without such bacteria (Table IV) This difference was not statistically significant ($p>0\ 05$)

C1q binding substances Fifty seven sera from patients with chronic OME were tested for C1q binding substances With C1q DT sera from OME patients showed significantly higher amounts of C1q binding substances than sera from the controls C1q reacting substances corresponding to more than 100 µg aggregated IgG per ml were present in 16 of 57 sera from OME patients and in 1 of 29 sera in the control group ($p<0\ 01$) Occurrence of

Table III *Frequency of C1r–C1s complexes and increased levels of C$\bar{1}$r–C1s–C$\bar{1}$ IA complexes in sera obtained from 86 patients with chronic OME and from 29 healthy children*

	Frequency of C1 subcomponent complexes in sera of	
	patients	healthy children
Increased level of C$\bar{1}$r–C$\bar{1}$s–C$\bar{1}$ IA complexes*	20/86	0/29
Presence of C1r–C1 complexes	35/86	3/29

More than 25% of ... and Method

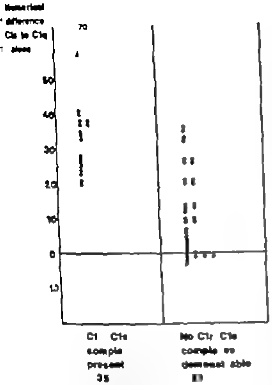

Fig. 1. Numerical differences between C1s and C1q serum concentrations in chronic OME patients in relation to presence of C1r–C1s complexes.

C1q binding substances in patient sera could not be correlated to presence of pathogens in the nasopharyngeal specimens ($p>0.05$, Table IV).

When the same sera were tested for C1q binding substances using the C1q BA, there was no difference between controls and OME patients.

C1q binding substances, estimated by C1q DT, were present in 3 % of the OME sera containing C1r–C1s complexes as compared with 19% of the OME sera where no such complexes were present. This difference was not statistically significant, $p>0.05$.

In serous MEE, C1q binding substances detected by C1q DT and/or C1q BA were found in 17 of 20 samples tested. These values ranged from 130 µg/ml to > 000 µg/ml.

In addition, C1q binding substances were demonstrated in the serum of 6 of these 20 patients and only when such substances were present in MEE.

The presence of C1q binding substances in MEE could not be correlated to the occurrence of pathogenic bacteria in the nasopharynx. High levels of C1q reactive substances (540 µg/ml, respectively >2000 µg/ml) were demonstrated in 2 patients where no pathogens were isolated from the nasopharynx.

II. Follow-up examination of patients with chronic OME

C1 subcomponent complesex and C1q binding substances. Blood was sampled from 31 patients who were re-investigated 4 weeks after the first myringotomy and aspiration of MEE. When microscopic otoscopy demonstrated retracted tympanic membranes and/or MEE, a second myringotomy was performed.

MEE was again present in 11 patients (group I). The other 20 patients did not demonstrate MEE (group II), although some of them had retracted tympanic membranes. In both groups C1r–C1s complexes and increased amounts of $\bar{C1r}$–$\bar{C1s}$–$\bar{C1}$ IA complexes were less frequently found in the follow-up serum samples (Table V). In the patients without MEE at follow-up, however, the decrease in $\bar{C1r}$–$\bar{C1s}$–$\bar{C1}$ IA complexes was most pronounced ($p<0.001$).

At follow-up, the C1q DT revealed unchanged serum levels in 41 %, increased levels

Table IV. *Occurrence of C1r–C1s complexes and C1q binding substances in sera correlated to bacteriological finding in nasopharynx in patients with chronic OME*

Bacteria in nasopharynx	Frequency of C1r–C1s complexes	C1q DT >100 µg/ml
S. pneumoniae	6/9	–/5
H. influenzae	9/18	4/15
B. catarrhalis	9/18	6/13
More than one pathogen	8/?7	3/18
No pathogens	3/14	1/6

Estimated with C1q DT. Levels greater than 100 µg/ml are regarded positive.

Table V *Frequency of C1r–C1s complexes and elevated levels of C1r–C1s–C1 IA complexes in sera from patients with chronic OME at first myringotomy and at the 4 week follow-up*

	C1r–C1s complexes		C1r–C1s–C1 IA complexes	
	First sample	Follow-up sample	First sample	Follow-up sample
Group I (MEE present at follow-up)	6/11	3/11	4/11	3/11
Group II (MEE absent at follow-up)	8/20	4/20	8/20	1/20

in 37% and decreased levels in 22% of the patients

DISCUSSION

The mean ages for patients in the OME group (3 9 years) and the control group (6 4 years) did differ ($p<0\ 05$) However all the differences described between these groups remained unaltered when only OME patients 4 years or older ($n=47$ mean 5 9 years) were included

The presence of C1r–C1s–C1 IA complexes in normal sera indicates C1 activation (Laurell et al 1978) The elevated levels of such complexes in serum from patients with chronic OME implies increased C1 activation also in the absence of manifest hypocomplementemia C1 can be activated by immune complexes by CRP in complex with various polyanion/cations (Siegel et al 1975) and with pneumococcal C-polysaccharide (Kaplan & Volanakis 1974 Claus et al 1977) and by enzymes such as plasmin and trypsin (Ratnoff & Naff 1967) The present investigation does not however elucidate the nature of the C1 activating substances appearing in OME which are evidenced by increased C1r–C1s–C1 IA level

An impressive finding was the high amounts of C1r–C1s complexes present in 41% of patients with chronic OME the information of which has not yet been explained The relative excess of C1r and C1s compared with C1q, may be the result of an acute phase reaction However normal levels of the acute phase protein CRP registered in OME patients does not sustain this possibility A dissociation equilibrium of C1 subcomponents in normal serum has recently been reported (Bartholomew & Esser 1977) As it is known that some bacterial components bind C1q directly (Loos et al 1974) it can be speculated that such binding may cause a disequilibrium between the C1 subcomponents which leads to the formation of C1r–C1s complexes

C1q binding substances exceeding 100 μg/ml were demonstrated in the sera of 28% of patients by C1q DT although the C1q BA was negative Various bacterial products may be detected by the C1q DT (Sobel et al 1975), but probably not by the C1q BA (Zubler et al 1976) whereas immune complexes are detected by both tests (Sobel et al 1975 Zubler et al 1976) Thus the C1q binding capacity demonstrated in OME sera is possibly not due to immune complexes but other C1q reactive substances High amounts of C1q binding substances were detected in MEE using both the C1q BA and C1q DT indicating the presence of immune complexes

Maxim et al have demonstrated immune complex-like substances in serous MEE and proposed that they were composed of anti

bodies and bacterial antigens derived from the nasopharynx (1977). In the present investigation, immune complexes were demonstrated in MEE from 2 patients where no pathogenic bacteria could be isolated from the nasopharynx. This finding does not, however, exclude that antigens participating in the formation of immune complexes are or have been present in the nasopharynx.

At the 4-week follow-up all but one of the patients in whom MEE was not present displayed $C\bar{1}r$–$C\bar{1}s$–$C\bar{1}$ IA levels within normal range, while C1r–C1s complexes had disappeared in 4 of 8 patients. It was, however, not possible in the present study to demonstrate in sera correlation between decreased concentrations of C1q binding substances and of $C\bar{1}r$–$C\bar{1}s$–$C\bar{1}$ IA complexes or the disappearance of C1r–C1s complexes.

Besides activation of C by the classical pathway, we have also found decreased levels of properdin in chronic OME patients which may indicate that complement is activated by the alternative pathway as well. No further attempts were made in the present study to search for other parameters to support this latter possibility.

In conclusion, the presence of immune complexes in MEE and of C1q reactive substances in serum supports the concept that the inflammatory process in chronic otitis media may be sustained by an immunological process.

ZUSAMMENFASSUNG

Das Auftreten und die Quantität von Immunkomplexen im Mittelohrexsudat und Serum sowie das Serumniveau des Komplements bei Patienten mit chronischer Otitis media wurden untersucht. Immunkomplexe wurden in [illegible] in serösem Mittelohrexsudat und in [illegible] in den Sera nachgewiesen. Verminderte C1q-Werte und das Vorkommen von anormalen Komplexen, zusammengesetzt aus Untereinheiten des ersten C-Faktors, hat auf eine gestörte Funktion des Komplement-Systems hingewiesen. Erhöhte C-Aktivierung durch den klassischen Aktivierungsweg wurden bei [illegible] der Patienten nachgewiesen. Vermindertes Niveau von Properdin wurde auch beobachtet. Nach Abklingen der Otitis hatten die Veränderungen des Komplement-Systems eine Tendenz zur Normalisierung.

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Karin Prellner M.D
Department of Otorhinolaryngology
University Hospital of Lund
S 221 85 Lund
Sweden

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EFFECTS OF TOPICAL USE OF β-ADRENOCEPTOR STIMULANTS ON NASAL MUCOSA. RHINOMANOMETRIC EVALUATIONS IN EXPERIMENTS WITH TERBUTALINE AND KWD 2131

I. *Normal Persons and Hay Fever Patients in Asymptomatic Period*

Gunnar Svensson, Birgitta Hegardt and Thorvald Löfkvist

From the Department of Otorhinolaryngology, University Hospital, Lund, Sweden and Medical Department of AB Draco, Lund, Sweden

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Abstract. The effects on nasal airway resistance of the β-adrenoceptor stimulants terbutaline (Bricanyl®) and KWD 2131 intranasally have been studied by means of posterior rhinomanometry in 16 healthy volunteers and 13 asymptomatic patients with allergic rhinitis. The study was performed as a randomized, placebo-controlled, double-blind crossover trial. No obvious changes in nasal resistance were revealed. This makes the nasal mucosa a suitable organ for testing the capacity of β-adrenoceptor stimulants to reduce the release of mediators from mast cells after allergen challenges.

The autonomous nervous system plays an important part in the regulation of tone in the nasal vascular bed. This has been shown in studies performed after sympathetic nerve stimulation and cervical sympathectomy and is also reflected by the successful clinical use of α-adrenoceptor stimulating drugs (Malcomson, 1959; Malm, 1973; Änggård & Densert, 1974).

It is well established that α-adrenoceptors predominate in the nasal vascular bed of man as well as of several animal species but opinions about the existence of β-adrenoceptors differ (Hall & Jackson, 1968; Malm, 1974; Hiley et al., 1978). A sparse distribution of β-adrenoceptors in the vessels of the human nasal mucosa cannot be excluded (McLean et al., 1976).

An anti-allergic effect is also ascribed to β-adrenoceptor stimulating drugs, i.e. they reduce mediator release from mast cells after allergic provocation (Assem & Schild, 1969; Ishizaka et al., 1971; Kaliner & Austen, 1975). In patients with allergic rhinitis such an effect on the mast cells might imply reduced nasal congestion after allergic challenges and in periods of allergic symptoms.

The aim of this investigation was to evaluate any effect of β-adrenoceptor stimulants applied locally to the nasal mucosa, since the results of earlier studies on animals as well as humans are contradictory (Hall & Jackson, 1968; Malm, 1974; McLean et al., 1976; Hiley et al., 1978). Two trial groups—normal subjects and patients with seasonal allergic rhinitis—were studied, as a potential difference between them in sensitivity of possible β-adrenoceptors may exist, i.e. a quality of the nasal vessels similar to that of the bronchial muscles according to the hypothesis of Szentivanyi (1968). The obtained results could also be used as a background for an evaluation of the effect on mast cells of the tested substances in patients with allergic rhinitis in a consecutive study (Svensson, 1980).

MATERIAL AND METHODS

The study involved a total of 29 subjects (16 normal persons and 13 patients with allergic rhinitis). The normal volunteers (7 males and 9

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MATERIAL AND METHODS

The study involved a total of 29 subjects (16 normal persons and 13 patients with allergic rhinitis). The normal volunteers (7 males and 9

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females) aged 24–38 years (average 31 years) had never had symptoms simulating allergic rhinitis dermatitis urticaria or bronchial asthma and had no heredity for these diseases Analysis of IgE/serum with PRIST was within normal limits The patients with seasonal allergic rhinitis (7 males and 6 females) aged 17–29 years (average 25 years) were asymptomatic during the trial All of them were sensitive to pollen as shown by positive skin test and positive provocation test and had suffered from hay fever during the last two seasons at least

The preparations under investigation were two β adrenoceptor stimulating drugs terbutaline sulphate (Bricanyl®) dihydroxy phenyl ethanol sulphate (KWD 2131) and placebo (isotonic saline solution) Terbutaline 0.5 and 5 mg and KWD 2131 1 25 and 5 mg were administered to each nasal cavity The dosage of terbutaline sulphate was based on experiences from treatment of asthmatic patients (McPhillips 1977) from a high dosage tolerance study (Bennis & Svedmyr 1977) and from a study of patients with allergic rhinitis performed in our laboratory (Hegardt & Svensson 1977) The doses of KWD 2131 were based on results from studies on the protective property of nebulized (Hegardt et al 1978) and subcutaneously (Pegelow & Strandberg 1978) administered KWD 2131 in patients with asthma challenged with allergen and histamine The drugs were applied locally as nose drops from a pipette in two equal portions (2×0 08 ml) to each nasal cavity with a time interval of 2 minutes between the portions

During the drug application the subject was sitting with his/her head tilted backwards and laterally for administration against the lower turbinate Placebo was delivered in the same volume and manner as terbutaline and KWD 2131 After each application the subject was questioned regarding any taste of drug for evaluation of nasal and/or pharyngeal deposition Experiments with suspected pharyngeal deposition of the drugs were put off to some other day When used the drugs had the same temperature as the testing room, i.e 22 23°C In the meantime they were stored in a refrigerator (4–8°C)

The study comprising six visits was of a double-blind crossover type and was conducted during a pollen-free period (January–March) in 1978 All subjects were asymptomatic as regards nasal symptoms and were free from drugs The intervals between testing each preparation were at least 2 days. The drugs were tested in a randomized order The nasal patency for both nasal cavities together (total nose) was determined during spontaneous breathing as posterior rhinomanometry

Rhinomanometry

The nasal air flow was measured with a pneumotachograph attached to an ordinary narcosis mask (type Dräger) covering the nose and mouth The pressure difference across the nose was estimated by recording pressure in the oropharynx through a catheter held between teeth with lips closed and placed with its open tip in the oropharynx and simultaneously recording pressure in the mask external to the nares Through electronic transducers the pressure difference was displayed on the Y axis of a memory-oscilloscope screen and the flow on the X-axis From standardized points of the pressure-flow curve *ad modum* Broms et al (1979) a mathematical description was obtained for each curve From this description an angle v_2 between the curve and the flow axis was calculated Increasing values of v_2 denote higher nasal airway resistance Differences between curves expressed as differences of v_2 were analysed statistically with Student s paired t test A proper value of nasal resistance out of v_2 is, for a total nose $R=5 \tan v_2$

Prior to and after each test procedure the rhinomanometric equipment was calibrated in two steps Firstly the deflections of pressure changes were adjusted with a stooping manometer and secondly the deflections of com

bented flow-pressure changes by means of a flow over a known resistance.

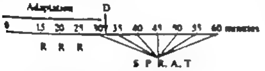
The subjects were in a sitting position throughout the testing procedure. The first rhinomanometric determination was done after 15 minutes adaptation to the testing room and repeated every 5 min. The preparations were administered after 30 minutes stay in the testing room.

The condition of the nose was also recorded rhinoscopically as to the presence of oedema and secretion on a 0–3 scale (0 = no symptoms, 1 = mild, 2 = moderate, 3 = severe) before and every 5 min after application of the preparations. This examination was performed after the rhinomanometric evaluation. Also the patients subjective opinion of nasal symptoms was recorded on a 0–3 scale. The pulse frequency was regularly determined before drug application and also before rhinomanometry. Any hand tremor before and after drug administration was also recorded on a 0–3 scale.

Because of acclimatization of the subject to the experimental situation no statistical analyses were done until after 30 minutes stay in the testing room.

The test procedure is given schematically as follows.

* Visits 1–6



D drug application
S subjective evaluation
P pulse-frequency determination
R posterior rhinomanometry
A anterior rhinoscopy
T hand tremor examination

* At rest no drug was applied and the subjective evaluation, pulse frequency determination, rhinoscopic and hand tremor examination were excluded.

For the allergic patients the effect of the lower doses of terbutaline, KWD 2131 and placebo was only followed for 15 min, as 0.5 mg terbutaline was without any obvious effect in a previous pilot study (Hegardt & Svensson 1977).

The study was approved by the Ethical Committee of the University of Lund and informed consent was obtained from all patients.

RESULTS

Fig. 1 shows the mean basal nasal airway resistance (expressed as the angle v_g) in the healthy volunteers and asymptomatic patients with allergic rhinitis in a sitting position. The values were somewhat higher for the allergic patients than for the normal volunteers, but these differences are not statistically significant. The variations of nasal airway resistance during 60 minutes observation are small and without obvious differences between the groups.

Application of placebo to the nasal mucosa induced minor increases in the mean nasal airway resistance in both groups, but these increases were not statistically significant (Figs. 2 and 3). Terbutaline, 0.5 mg, and KWD 2131, 1.25 mg, evoked changes in the nasal airway resistance of about the same degree as placebo in both groups. Terbutaline, 5 mg, and KWD 2131, 5 mg (Figs. 2 and 3), induced a more marked increase in nasal airway resistance in both the healthy and allergic persons. These elevations were still small but statistically significant when they were analysed for each drug. However, in comparison with placebo there was neither any evident effect of terbutaline nor of KWD 2131 (Table I). The participants experienced no nasal changes during the experiments and the rhinoscopic examination revealed no alterations.

In the high dose, terbutaline induced tachycardia and tremor in all subjects. The pulse frequency increased as early as 5 min after application of the drug and remained increased during the whole experiment. The average increase was about 15–30 beats per minute. Tremor was noticed 5 min after application of the drug, but was pronounced after 10 min and for the rest of the experiment. No other side effects were discovered.

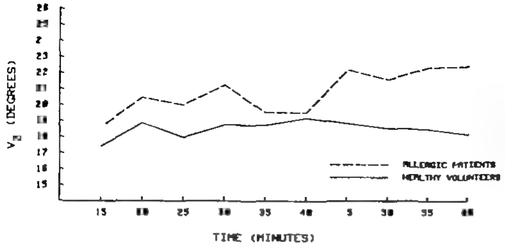


Fig 1 Nasal airway resistance versus time Mean values of 16 healthy volunteers and 13 patients with allergic rhinitis, respectively

DISCUSSION

The basic aim of this investigation was to evaluate any effect of β adrenoceptor stimulants locally applied to the nasal mucosa as earlier studies on animals as well as humans are contradictory In experiments on dogs Hall & Jackson (1968) could not demonstrate β-adrenoceptors in the nasal blood vessels In cats Änggård & Edwall (1974) found no β adrenoceptors but Malm (1974) has demonstrated such receptors in the resistance vessels and Hiley et al (1978) in the capacitance vessels The techniques (endotracheal tube intra-arterial injections cervical sympathic trunk dissection and transection surgical exploration of the pterygomaxillary fossa) used in these experiments could not be used in humans

In three studies on humans (Grobler 1966 Cohen 1970 McLean et al 1976) isoprenaline—a powerful stimulant of β_1- as well as β_2-adrenoceptors—has been used with differing results Grobler (1966) reported that the drug caused increased nasal airway resistance but Cohen (1970) found no effect and McLean et al (1976) registered a somewhat variable response with in most instances a greater increase than after saline application Methodological variations (different subject groups, various forms for administration of drugs dissimilar types of equipment for measurement of nasal airway resistance and varying time points for this registration) make a comparison of the results of the three studies very difficult

The study by McLean et al (1976) can in several respects be compared with the present

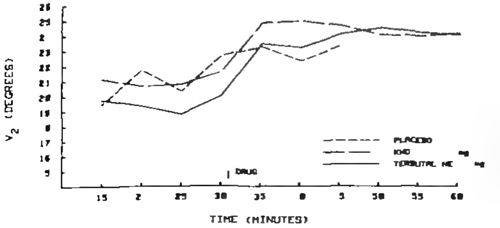
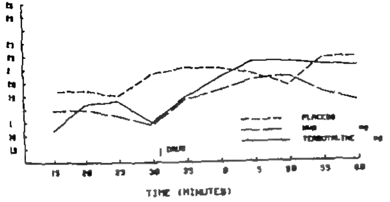


Fig 2 Nasal airway resistance versus time. Mean values of 13 patients with allergic rhinitis.



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investigation. Both deal with normal subjects and asymptomatic patients with hay fever and make use of posterior rhinomanometry. The studies differ however with regard to the manner of drug application, to points of time for measurements and to the methods for calculation of nasal airway resistance. These differences might contribute to the difference in results. Moreover, McLean et al (1976) used isoprenaline, while the present study was performed using terbutaline and KWD 2131, two substances with a more selective β_2-adrenoceptor effect. Therefore the differing characters of the drugs could also explain the non-corresponding results.

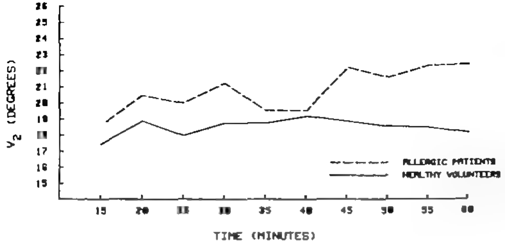
The method with topical application of substances presupposes local absorption of drugs. The nasal vascular bed seems to have a high capacity for absorption. Judging from the side effects—tachycardia and tremor—in the present experiments with terbutaline 5 mg to each nasal cavity, this is in fact the case. Thus β-adrenoceptors, if present in the human nasal vessels, should have been stimulated.

When β-adrenoceptors are stimulated, blood vessels commonly dilate. If the capacitance vessels in the human nasal mucosa contain β-adrenoceptors, their stimulation would evoke vasodilatation leading to increased nasal airway resistance, while a dilatation of the resistance vessels increases the blood flow with comparatively smaller changes in nasal

Table 1 *Comparison of the increase in nasal airway resistance measured a[illegible] relative to the 30 min [illegible]*

Minutes / Drug applied	35 Increase in	40	45	50	55	60
Healthy person						
Terbutaline 5 mg placebo	1 4	2 9	4 5*	5.3	3 1 n s.	2.9 n s.
Terbutaline 5 mg no appl	1 9	2 9	4 4	4 6	4 4	4 6
KWD 2131 5 mg placebo	1 4	2	3 4	4 5	1.2	0.5
KWD 2131 5 mg no appl	1 9	2 1	3 3	3 8*	4.6	3.3 s.
KWD 2131 1 25 mg no appl	2 4	0 7	1 3	2 3 n	3 8*	3 1 n.s.
Allergic patient						
Terbutaline 5 mg placebo	1 3	5 0*	3 9 s.			
KWD 2131 5 mg placebo	1 9	4	2.3 n			

*0 05 ** $p<0.01$ Analysis not possible due to no registration of the effect of placebo



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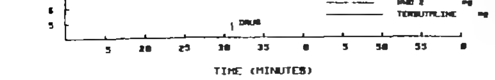

Fig 2 Nasal airway resistance versus time Mean values of 13 patients with allergic rhinitis.

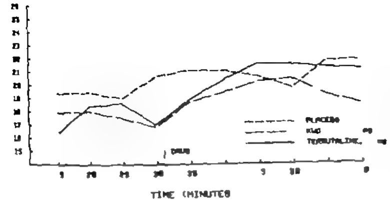

Fig. 3. Nasal airway resistance versus time. Mean values of 16 healthy volunteers.

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Table I. *Comparison of the increase in nasal airway resistance, measured as relative to the 30 min value*

Minute Drug compared	35 Increase in	40	45	50	55	60
Healthy persons						
Terbutaline 5 mg placebo	1 4	2.9 s.	4.5**	5.3	3.1 s.	2.9 n.s.
Terbutaline 5 mg no appl	1 9 s.	2.9 n.s	4 4	4.6	4 4	4 6
KWD 2131 5 mg placebo	1 4	2	3 4	4.5**	1.2	0.5 n.s.
KWD 2131 5 mg no appl	1 9	1 s.	3.3	3.8	.6	2 3 n.s.
KWD 2131 1 25 mg no appl	2 4 s.	0.7	1 3 n.s	3 s.	3.8*	3 1 n.s.
Allergic patient						
Terbutaline 5 mg placebo	1 3 s.	5 0*	3 9			–
KWD 2131 5 mg placebo	1 9 n.s	4	2.3		–	–

not significant * = p<0.05 ** = p<0.01 – = Analysis not possible due to no registration of the effect of placebo

congestion In the present study subjective experiences and rhinoscopic and rhinomanometric results after nasal application of the β-adrenoceptor stimulating drugs used both in normal persons and in hay fever patients showed only minor variations compared with the administration of placebo These results indicate none or only few β-adrenoceptors of functional significance in the nasal mucosa, at least in the capacitance vessels In consequence of this a defect β-receptor function like that of asthmatic patients according to the hypothesis of Szentivanyi (1968) could neither be established nor rejected in hay fever patients

In hay fever patients nasal provocations with appropriate allergens induce swelling of the nasal mucosa and higher nasal airway resistance due to the release of mediators from the mast cells β-adrenoceptor stimulants are shown to inhibit the antigen-induced release of histamine in many tissues For the nasal mucosa, treatment with β-adrenoceptor stimulants before nasal challenges would mean reduced swelling of the mucosa and in consequence of this decreased nasal airway resistance Thus the absence of demonstrable vascular effects in the nasal mucosa after application of β-adrenoceptor stimulants as shown in this investigation makes the nasal mucosa a suitable organ for testing the effect of β adrenoceptor stimulating agents on mast cells

ZUSAMMENFASSUNG

Die Wirkung der intranasalen β-adrenoceptorstimulierenden Substanzen Terbutaline (Bricanyl®) und KWD 131 auf den Atemwiderstand der Nase wurde mit Hilfe der posterioren Rhinomanometrie an 16 gesunden freiwilligen Versuchspersonen und 13 asymptomatischen Patienten mit allergischer Rhinitis untersucht. Der Test wurde als ein doppelter Blindversuch unter Placebokontrolle durchgeführt. Es wurden keine Veränderungen im Atemwiderstand der Nase festgestellt. Dies zeigt, daß die nasale Schleimhaut ein brauchbares Organ für die Testung der Fähigkeit von β-adrenoceptorstimulierenden Substanzen ist um die Freilassung der Mediatoren von Mastzellen nach allergischen Provokationstesten zu reduzieren.

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Gunnar Svensson M D
Dept. of Otorhinolaryngology
University Hospital
S-221 85 Lund
Sweden

congestion In the present study subjective experiences and rhinoscopic and rhinomanometric results after nasal application of the β-adrenoceptor stimulating drugs used both in normal persons and in hay fever patients showed only minor variations compared with the administration of placebo These results indicate none or only few β-adrenoceptors of functional significance in the nasal mucosa, at least in the capacitance vessels In consequence of this a defect β-receptor function like that of asthmatic patients according to the hypothesis of Szentivanyi (1968) could neither be established nor rejected in hay fever patients

In hay fever patients nasal provocations with appropriate allergens induce swelling of the nasal mucosa and higher nasal airway resistance due to the release of mediators from the mast cells β-adrenoceptor stimulants are shown to inhibit the antigen-induced release of histamine in many tissues For the nasal mucosa treatment with β-adrenoceptor stimulants before nasal challenges would mean reduced swelling of the mucosa and in consequence of this decreased nasal airway resistance Thus the absence of demonstrable vascular effects in the nasal mucosa after application of β-adrenoceptor stimulants as shown in this investigation makes the nasal mucosa a suitable organ for testing the effect of β-adrenoceptor stimulating agents on mast cells

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lated. Regional blood flow resistance was expressed as the ratio of the perfusion pressure to the venous blood flow Induced changes of flow resistance were expressed as per cent of the resting flow resistance

To record changes of tone in the capacitance vessels a thin-walled waterfilled balloon was placed in the nasal cavity on the side where the vein was cannulated Pressure changes in this balloon are directly related to volume changes in the nasal mucosa, and volume changes occurring shortly after a vascular smooth muscle stimulus can be considered to reflect changes in the tone of the nasal capacitance vessels (Malm 1974) Induced pressure changes were expressed in cmH_2O above the resting pressure in the balloon.

Highly purified porcine VIP dissolved in 0 9% NaCl solution was given as retrograde infusions through a small polyethylene catheter in the lingual artery VIP was tested over a wide range of doses the material to be presented refers to the following: 1.7×10^{-10} 3×10^{-10} 5.3×10^{-10} 9×10^{-10} and 17×10^{-10} mol/kg b wt × min. The infusions (0 008–0 08 ml/min) lasted for 30 to 120 sec. Saline infusions at the same rates served as controls The effects of VIP were also analysed before and after atropine (1 mg/kg b wt.) was given i v in 2 cats.

RESULTS

Infusions of VIP in doses from 1.7×10^{-10} mol/kg b wt. × min increased the venous blood flow and the pressure in the balloon Fig 1 illustrates such effects in response to successively increasing doses from 1 7 to 9×10^{-10} mol/kg b wt × min VIP caused a transient dose-dependent increase in blood flow and balloon pressure At the same time the systemic arterial blood pressure decreased, especially with the larger doses of VIP Nasal vascular resistance calculated at the time of the peak blood flow increase decreased by

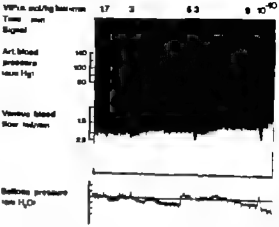

Fig 1 Cat, 4.8 kg. Effects of i.a. infusions of VIP on systemic arterial blood pressure nasal blood flow and balloon pressure in the nasal cavity

9% 12% 25% and 36% for the four different doses given Balloon pressure increased simultaneously by 0 1 0.3 1.2 and 1 4 cmH_2O indicating graded dilator responses in the capacitance vessels. The decreases in the arterial blood pressure are most probably caused by systemic effects of VIP although the infusions were given close arterially Since a blood pressure fall may lead to passive elastic collapse of the vessels data were included only when the systemic blood pressure did not fall below 60 mmHg upon VIP infusion

Fig. 2 summarizes the results with regard to the dilator effects on the resistance vessels calculated at the time of the peak blood flow (*cf* Fig 1) VIP also dilated the nasal capacitance vessels, as evidenced by the graded pressure increases in the balloon (Fig. 3)

When VIP in the same dose was given repetitively at different time intervals no signs of tachyphylaxia were observed

The dilator effects of VIP on the resistance and capacitance vessels were not affected by atropine administration

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VIP was kindly supplied by Professor V Mutt, Department of Chemistry Karolinska Institutet, Stockholm, Sweden.

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EFFECTS OF VASOACTIVE INTESTINAL POLYPEPTIDE ON RESISTANCE AND CAPACITANCE VESSELS IN THE NASAL MUCOSA

L. Malm F Sundler and R Uddman

From the Department of Otolaryngology University Hospital Malmö and Departmen of Histology University of Lund Lund S eden

(Received October 26 1979)

Abst act Vasoactive intestinal polypeptide (VIP) immunoreactive nerves have previously been demonstrated in the feline nasal mucosa. In the present investigation it is shown that VIP given close arterially dilates both resistance and capacitance vessels in the nose of the cat This vasodilatation is atropine resistant It is suggested that VIP serves a physiological role in the neurogenic control of blood vessels in the nasal mucosa.

Immunohistochemical studies have revealed a wide distribution of nerves containing vasoactive intestinal polypeptide (VIP) in the periphery (Bryant et al 1976 Larsson et al 1976 Alm et al 1977 Larsson et al 1977 Sundler et al 1978 Uddman et al 1978*a*) Available evidence suggests that peripheral VIP nerves form part of the autonomic nervous system and that VIP serves a neurotransmitter or modulator role (Giachetti et al 1977 Emson et al 1978 Fahrenkrug et al 1978)

Effects of exogenous VIP on blood vessels have been investigated in various species and tissues Generally the results have been expressed as changes in blood flow or in vascular tone or resistance (Said & Mutt 1970 Thulin & Olsson 1973 Kachelhoffer et al 1974 Edvinsson & McCulloch 1979 Shimizu & Taira 1979) Such changes however do not register events in all parts of the vascular bed A more complete and functional way of investigating the effects of vasoactive agents is to study changes of tone in both resistance and capacitance vessels (cf Mellander & Johansson 1968)

The aim of the present investigation was to study the effects of VIP simultaneously and separately on resistance and capacitance vessels As a model tissue we have chosen the nasal mucosa of the cat since methods have previously been developed for studies in this tissue (Malm 1974) and since numerous VIP nerve fibres surround nasal blood vessels and seromucous glands (Uddman et al 1978*b* 1979)

MATERIAL AND METHODS

Six adult cats (3 0–4 8 kg) were anaesthetized with α-chloralose (80 mg/kg) intravenously after induction with diethylether The cats were breathing spontaneously through a tracheal cannula The body temperature was kept constant at 37°C with the aid of a thermostatically heated table The autonomic reflex activity in the nasal vessels was reduced by severing the Vidian nerve and the cervical sympathetic nerve

In order to record changes of tone in the resistance vessels of the nasal mucosa the following procedures were used (for details, see Malm 1974) The pterygopalatine vein on one side was cannulated via a transpalatinal approach and the venous blood flow was measured with an optical blood flow recorder unit. The height of the venous outflow catheter was set to about 6 cm above the pterygopalatine foramen The arterial blood pressure was continuously recorded via a catheter in the right femoral artery The perfusion pressure (the difference between arterial blood pressure and venous blood pressure) could then be calcu

lated. Regional blood flow resistance was expressed as the ratio of the perfusion pressure to the venous blood flow Induced changes of flow resistance were expressed as per cent of the resting flow resistance

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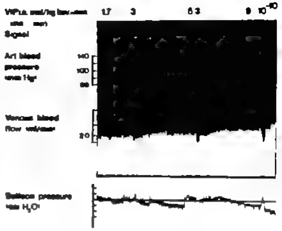

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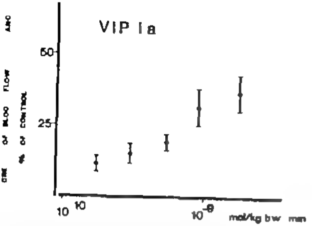

Fig 2 Changes in nasal blood flow resistance in per cent of control after i.a infusions of different doses of VIP to 6 cats Mean changes ± S E M are given

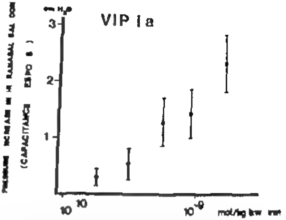

Fig 3 The simultaneous changes of the tone of the nasal capacitance vessels expressed in cmH_2O above control values after the same doses of VIP as in Fig 2 Mean values ± S E.M are given.

DISCUSSION

The blood vessels in a tissue can functionally be divided into precapillary and postcapillary resistance vessels precapillary sphincters exchange vessels (capillaries) and capacitance vessels (Mellander & Johansson 1968) In some tissues such as the nasal mucosa, shunt vessels also exist (Änggård 1974*a*) The main resistance function is confined to the small arteries and arterioles and the tone of these vessels therefore is the main determinant of blood flow In the nose the capacitance vessels consist mainly of sinusoids and veins (Malm 1974) The tone of these vessels is therefore the major determinant of the blood content in the nasal mucosa hence serving as an important modulator of mucosal volume

Several studies have shown that VIP is a potent dilator of resistance vessels in various tissues such as the intestine salivary glands and pia mater (for references see introduction) In the present study it was found that VIP given intra-arterially caused a dose dependent and simultaneous dilatation of both resistance and capacitance vessels in the nasal mucosa

VIP is known to cause secretion from certain exocrine glands (Said & Mutt 1970 Barbezat 1973 Konturek et al 1976 Domschke et al 1977) Therefore some of the registered changes in balloon pressure in the nasal cavity might have been due to nasal secretion. Since the balloon pressure however usually returned within a short time to the control level between repetitive infusions (see Fig 1) nasal secretion does not seem to be the cause of the increase in balloon pressure in the present experiments

Electrical stimulation of nerves to certain blood vessels evokes a dilatation which is resistant to atropine (Hilton & Lewis 1955 Bhoola et al 1965 Schachter 1969 Gautvik 1969) Such an atropine resistant vasodilatation is also evoked in the feline nasal mucosa, when the Vidian nerve is stimulated (Malm, 1973 Änggård 1974*b*) Blood vessels in the nasal mucosa are richly supplied with VIP-containing nerve fibres (Uddman et al 1978*b* 1979) and preliminary experiments have shown that stimulation of the Vidian nerve leads to release of VIP (Uddman et al 1980) simultaneously with a vasodilatation The present demonstration that infusions of VIP cause an atropine resistant dilatation of nasal blood vessels therefore suggests that VIP might serve a physiological purpose in the control of vascular resistance and capacitance in the nasal mucosa

ACKNOWLEDGEMENTS

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ZUSAMMENFASSUNG

Nerven die VIP (vasoaktive intestinale polypeptide) Immunoreaktivität aufweisen, konnten vor kurzem in der Nasenschleimhaut von Katzen nachgewiesen werden. In dieser Arbeit wird gezeigt, daß VIP gegeben als arterielle Infusionen, sowohl die Resistanz wie die Kapacitanzblutgefäße in der Nase der Katze erweitert. Diese Gefäßerweiterung wird nicht von Atropin beeinflußt. Es ist anzunehmen, daß die VIP Substanz bei der Kontrolle der Nasenschleimhaut eine physiologische Rolle spielt.

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Lars Malm M.D
University of Lund
ENT Department
Malmö General Hospital
S 214 01 Malmö Sweden

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ARTHUS TONSILLITIS IN THE RABBIT

Histological Findings and Fibrinolytic Activity in the Blood

Tadayoshi Kosugi Tamotsu Morimitsu Osamu Matsuo and Hisashi Mihara

From the Department of Physiology and Otorhinolaryngology Miyazaki Medical College Miyazaki, Japan

(Received September 18, 1979)

Abstract Arthus-type hypersensitivity was induced experimentally in the tonsils of rabbits. Histopathological studies were performed on the Arthus tonsillitis so produced, and estimations of the plasma fibrinolytic activity were made on the blood of these rabbits. The findings obtained by macroscopic inspection of the tonsil revealed significant bleeding and swelling. Furthermore the histopathological studies demonstrated bleeding and infiltration of leukocytes into various parts of the parenchyma and connective tissue surrounding the tonsil during the early stages of tonsillitis. From the results concerning certain parameters of the fibrinolytic system in the blood it was demonstrated that during the early stage of tonsillitis, the fibrinolytic activity increased and whole plasmin was consumed. Based on the above findings it seems that the change in fibrinolytic activity found in rabbits affected by Arthus tonsillitis closely resembles that in patients suffering from acute tonsillitis.

We previously reported that in human acute tonsillitis the fibrinolytic activity in the blood increased at the initial stage of onset (Kosugi et al 1979) However the mechanism of the increase in fibrinolytic activity could not be adequately explained Furthermore it was thought that the clinical progress of human acute tonsillitis might be modified by such increased fibrinolytic activity in the blood In order to clarify these problems Arthus-type hypersensitivity was induced experimentally in the tonsils of rabbits and histological investigations of the Arthus tonsillitis were undertaken in relation to the fibrinolytic activity in the blood of the rabbits The possibility that Arthus tonsillitis of the rabbit may provide a useful model for investigating the mechanism of increase in fibrinolytic activity in human acute tonsillitis is discussed

MATERIALS AND METHODS

Animals: normal healthy rabbits weighing 1.0–1.5 kg were used

Fibrinogen. Cohn Fraction I (Miles Laboratory) was dissolved in borate saline buffer pH 7.8)

Thrombin: bovine thrombin (Mochida Pharm Co) was dissolved in physiological saline

Urokinase (UK): Uronase (Mochida Pharm. Co) was dissolved in physiological saline

Streptokinase (SK): Varidase (Lederle Laboratories) was dissolved in physiological saline

Lysine–sepharose: sepharose 4B substituted by 1-lysine (Daiichi Pure Chemical Co) was used

Albumin: bovine serum albumin (BSA) (Seikagaku Kogyo Co) was dissolved in physiological saline

Adjuvant: complete Freund's adjuvant (Difco Laboratory) was used

Standard fibrin plates: standard fibrin plates were prepared according to the method of Astrup and Müllertz (1952)

Plasminogen-free fibrin plates: plasminogen free fibrinogen was prepared by affinity chromatography (Matsuda et al 1972 Matsumoto et al., 1973) and fibrin plates were then prepared according to the method of Astrup & Müllertz.

Induction of Arthus tonsillitis

A mixed solution of BSA (4 mg/ml) and complete Freund's adjuvant was injected intrader

Table I *Macroscopic findings after injection in non sensitized rabbits*

R. right tonsil L. left tonsil − unchanged + swelling, ++ hyperemia

Rabbit no	Tonsil R/L	Macroscopic findings Paren chyma	Tissue surrounding tonsil	Time after the injection
1	R	+	+	3 hr
	L	−	−	
2	R	+	−	3 hr
	L	−	−	
3	R	−	++	6 hr
	L	−	−	
4	R	+	++	6 hr
	L	−	+	
5	R	+	++	1 day
	L	−	−	
6	R	−	+	1 day
	L	−	−	
7	R	−	+	2 day
	L	−	+	
8	R	−	−	2 day
	L	−	+	
9	R	−	−	3 day
	L	−	−	
10	R	−	−	3 day
	L	−	−	
11	R	−	−	7 day
	L	−	−	
12	R	−	−	7 day
	L	−	−	

mally in the dorsal region of rabbits Two booster doses were given twice a week after the first injection The titers of antibody to BSA were estimated according to the precipitin test (Udaka 1975) at about the 7th week after the first injection Rabbits with a 2^4–2^6 titer were used in the subsequent experiments

Prior to macroscopic and histopathological observations 0 2–0.3 ml of BSA was injected into the right tonsil of the sensitized rabbits and the same volume of physiological saline was injected into the left tonsil Furthermore for comparison 0 2–0 3 ml of BSA was injected into the right tonsil of normal (non-sensitized) rabbits and 0.2–0 3 ml of physiological saline was injected into the left tonsil

For assessment of the fibrinolytic activity in the blood with and without Arthus tonsillitis the sensitized rabbits were divided into two groups In the Arthus tonsillitis group 0.2 0 3 ml of BSA solution was injected into the bilateral tonsils of the sensitized rabbits In the control group 0.2–0.3 ml of physiological saline was injected into the bilateral tonsils of the other sensitized rabbits

Macroscopic and histopathological observations of Arthus tonsillitis

The inflammatory findings in the parenchyma of the tonsils and pharyngeal tissue surrounding the tonsil were assessed macroscopically The appearance of swelling (+) hyperemia (++) bleeding (+++) and necrosis (++++) was judged macroscopically according to the criteria of Cochrane et al (1958) and Hayashi et al (1964) After completion of the macroscopic inspection the tonsil was excised and placed in 10% formalin solution The excised tonsil was stained with haematoxylin-eosin. The tonsil was divided into six portions viz. the lacunal epithelium interstitial portion of the parenchyma follicle blood vessels capsule and connective tissue surrounding the tonsil and these were studied histopathologically The degree of cell infiltration bleeding necrosis abscess and thrombus formation were evaluated in the various portions

Estimation of fibrinolytic activity in the blood

The plasma fibrinogen content was measured *ad modum* Quick (1969) The plasma euglobulin was sufficiently activated by a high volume of SK (1 000 U/ml) and the whole plasmin activity was measured on standard fibrin plates Plasma euglobulin was prepared *ad modum* Okamoto & Oshiba (1966) The content of plasma plasminogen was measured using affinity chromatography and plasminogen free fibrin plates that is the plasminogen obtained through lysine sepharose was activated with Uronase (50 U/ml) and the plasmin activity was estimated on fibrin plates prepared

from plasminogen free fibrinogen (Kosugi 1976) The inhibitory activity to urokinase of the plasma was estimated using the inhibitor fraction which was obtained from the plasma through lysine sepharose *ad modum* Urita et al (1973) The inhibitor fraction was mixed with an equal amount of Uronase (10 U/ml) and the mixture incubated at 37°C for 10 min. Then 0 03 ml portions of the mixture solution were dropped on standard fibrin plates. After incubation at 37°C for 20 hr the lysis areas were measured and the inhibitory activity of the inhibitor was expressed as an inhibitory ratio with respect to the lysis area of Uronase (5 U/ml)

RESULTS

1 *Macroscopic findings*

In the non-sensitized group (rabbits 1–12) soon after BSA injection into the tonsil slight swelling and hyperemia were observed However at 2 days after the injection these findings subsided (Table I) On the other hand in the sensitized group (rabbits 13–26) at 1–3 days after BSA injection into the right tonsil marked hyperemia and bleeding were observed On day 7 after the injection these findings subsided. In the left tonsil which received physiological saline only slight swelling and hyperemia were observed at an early stage (Table II)

Table II *Macroscopic findings after injection in sensitized rabbits*

R. right tonsil L. left tonsil, – unchanged + swelling, ++ hyperemia, +++ bleeding

Rabbit no	Tonsil	Macroscopic findings: Parenchyma	Macroscopic findings: Tissue surrounding tonsil	Time after the injection
13	R	+	+	3 hr
	L	+	–	
14	R	+	+	3 hr
	L	–	–	
15	R	++	++	6 hr
	L	–	–	
16	R	+	–	6 hr
	L	–	–	
17	R	+	++	1 day
	L	–	+	
18	R	+++	+++	1 day
	L	–	+	
19	R	++	+++	1 day
	L	–	++	
20	R	++	+++	2 day
	L	–	+	
1	R	+	++	2 day
	L	–	+	
22	R	++	+++	2 day
	L	–	+	
[illegible]	R	++	++	3 day
	L	–	+	
24	R	–	+	3 day
	L	–	–	
25	R	–	–	7 day
	L	–	–	
26	R	–	–	7 day
	L	–	–	

2. *Histopathological findings*

At 6 hr after BSA injection in non-sensitized rabbits (nos. 4 and 5) moderate cell infiltration into the lacunal and other portions was observed Furthermore at 1 day after the BSA injection moderate cell infiltration into the follicle was observed but bleeding was not observed At 3 hr after BSA injection into the right tonsil of the sensitized rabbit slight bleeding and cell infiltration into the interstitial tissue and follicle were noted (no 13) but at 4 hr after the injection, marked cell infiltration into the interstitial tissue and moderate bleeding into the connective tissue surrounding the tonsil were observed (nos. 17–20) In addition to these findings slight or light necrosis was also seen in the connective tissue surrounding the tonsil (nos 27–32) On the other hand at 2–3 days after the injection of BSA moderate bleeding and cell infiltration were observed (nos 20 and 23) On day 7 after the injection, the above histopathological findings disappeared Soon after injection of physiological saline into the left tonsil slight infiltration of cells and bleeding were observed but on days 2–3 after the injection these findings subsided (Table III).

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R right tonsil L left tonsil – unchanged + swelling, ++ hyperemia

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1	R	+	+	3 hr
	L	–	–	
2	R	+	–	3 hr
	L	–	–	
3	R	–	++	6 hr
	L	–	–	
4	R	+	++	6 hr
	L	–	+	
5	R	+	++	1 day
	L	–	–	
6	R	–	+	1 day
	L	–	–	
7	R	–	+	2 day
	L	–	+	
8	R	–	–	2 day
	L	–	+	
9	R	–	–	3 day
	L	–	–	
10	R	–	–	3 day
	L	–	–	
11	R	–	–	7 day
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Table II *Macroscopic findings after injection in sensitized rabbits*

R. right tonsil L. left tonsil – unchanged + swelling, ++ hyperemia, +++ bleeding

Rabbit no	Tonsil	Macroscopic findings: Parenchyma	Tissue surrounding tonsil	Time after the injection
13	R	+	+	3 hr
	L	+	–	
14	R	+	+	3 hr
	L	–	–	
15	R	++	++	6 hr
	L	–	–	
16	R	+	–	6 hr
	L	–	–	
17	R	+	++	1 day
	L	–	+	
18	R	+++	+++	1 day
	L	–	+	
19	R	++	+++	1 day
	L	–	++	
20	R	++	+++	2 day
	L	–	+	
21	R	+	++	2 day
	L	–	+	
22	R	++	+++	2 day
	L	–	+	
23	R	++	++	3 day
	L	–	+	
4	R	–	+	3 day
	L	–	–	
25	R	–	–	7 day
	L	–	–	
26	R	–	–	7 day
	L	–	–	

2 *Histopathological findings*

At 6 hr after BSA injection in non-sensitized rabbits (nos 4 and 5) moderate cell infiltration into the lacunal and other portions was observed Furthermore at 1 day after the BSA injection moderate cell infiltration into the follicle was observed but bleeding was not observed At 3 hr after BSA injection into the right tonsil of the sensitized rabbit slight bleeding and cell infiltration into the interstitial tissue and follicle were noted (no 13) but at 4 hr after the injection marked cell infiltration into the interstitial tissue and moderate bleeding into the connective tissue surrounding the tonsil were observed (nos 17–20) In addition to these findings slight or light necrosis was also seen in the connective tissue surrounding the tonsil (nos 27–32) On the other hand at 2–3 days after the injection of BSA moderate bleeding and cell infiltration were observed (nos. 20 and 23) On day 7 after the injection, the above histopathological findings disappeared Soon after injection of physiological saline into the left tonsil slight infiltration of cells and bleeding were observed but on days 2–3 after the injection these findings subsided (Table III)

Table III *Histopathological findings*

R right tonsil L, left tonsil − unchanged ± slight + light ++ moderate +++ strong

Rabbit No	Tonsil	lacunal epithelium				interstitial tissue				follicle				capsule				blood vessel				connective tissue surrounding tonsil				
		desquamation	cell infiltration	bleeding	swelling	cell infiltration	bleeding	swelling	necrosis	cell infiltration	bleeding	necrosis	abscess	cell infiltration	swelling	necrosis	abscess	hyperemia	cell infiltration	swelling of wall	thrombus	cell infiltration	bleeding	swelling	necrosis	abscess
4	R	+	++	±	−	+	−	−	−	+	−	−	−	−	−	−	−	+	−	−	−	+	+	−	−	−
	L	−	−	−	−	+	+	−	−	−	−	−	−	±	−	−	−	−	−	−	−	−	−	−	−	−
5	R	+	+	−	−	+	+	−	−	++	−	−	−	−	−	−	−	+	−	−	−	+	+	−	−	−
	L	−	−	−	−	−	+	−	−	−	−	−	−	±	−	−	−	−	−	−	−	±	−	−	−	−
13	R	±	−	+	−	−	+	−	−	+	+	−	−	−	−	−	−	+	−	−	−	±	+	−	−	−
	L	−	−	−	−	+	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−	±	−	−	−	−
17	R	++	+	−	−	++	±	−	−	++	−	−	−	++	−	−	−	+	−	−	−	±	+	−	−	−
	L	−	−	−	−	+	−	−	−	++	−	−	−	−	−	−	−	±	−	−	−	−	+	−	−	−
18	R	++	++	+	±	+++	±	±	−	+	+	−	−	±	+	−	−	+	+	−	−	+	−	+	−	−
	L	±	−	−	−	+	±	−	−	+	−	−	−	±	−	−	−	+	−	−	−	−	++	−	−	−
19	R	++	++	+	−	++	++	+	−	++	+	−	−	++	−	−	−	+++	±	−	−	++	++	−	−	−
	L	−	−	−	−	±	+	−	−	−	±	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−
20	R	++	−	+	−	+	−	−	−	+	+	−	−	+	+	−	−	++	±	−	−	++	++	−	−	−
	L	−	−	+	−	−	+	−	−	+	−	−	−	+	−	−	−	±	−	−	−	−	+	−	−	−
23	R	++	++	+	−	+	+	−	−	+	−	−	−	+	±	−	−	−	−	−	−	+++	++	−	−	−
	L	±	−	−	−	±	−	−	−	+	−	−	−	+	−	−	−	+	−	−	−	+	−	−	−	−
27	R	±	++	−	−	++	+	+	−	+++	−	−	−	++	±	−	−	++	±	±	−	++	−	++	+	−
	L	−	−	−	−	+	±	−	−	+	−	−	−	+	−	−	−	±	−	−	−	−	−	−	−	−
28	R	−	−	−	−	+	++	++	−	+	++	−	−	+	±	−	−	±	−	−	−	+	−	+	±	−
	L	−	−	−	−	−	++	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
29	R	++	++	−	−	+++	+++	++	−	++	++	−	−	+++	++	+	−	+	−	−	−	++	−	+	±	−
	L	−	−	−	−	+	−	−	−	−	±	−	−	±	−	−	−	−	−	−	−	−	−	−	−	−
30	R	++	+	++	+++	+	++	±	−	+	++	−	−	−	−	−	−	++	−	−	−	−	−	−	−	−
	L	−	−	−	−	+	±	−	−	−	−	−	−	±	−	−	−	+	−	−	−	−	−	−	−	−
31	R	++	++	±	−	++	++	+	−	−	++	−	−	++	+	−	−	+	−	−	−	−	−	−	−	−
	L	−	−	−	−	±	+	−	−	−	±	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−
32	R	++	±	+	+	++	+++	+	−	±	++	−	−	−	−	−	−	++	−	−	−	−	−	−	−	−
	L	−	−	−	−	−	++	−	−	−	−	−	−	−	−	−	−	++	−	−	−	−	−	−	−	−

3 *Changes in fibrinolytic activity in blood*

At days 1–3 an increase in fibrinogen content slight decrease in plasminogen content and marked decrease in whole plasmin were observed in the Arthus tonsillitis group compared with the control group Furthermore in both the Arthus tonsillitis group and the control group at 7 days after the injection into the tonsil the values of these parameters returned to those before the injection On day 2 after the injection into the tonsil the fibrinogen content in the Arthus tonsillitis group (650 7±99 8 mg/dl) was significantly higher than that in the control group (410 1±75 8 mg/dl) ($p<0\ 001$) (Fig 1) while the whole plasmin activity in the Arthus tonsillitis group (0 mm²) was significantly lower than that in the control group (22 4±17.3 mm²) ($p<0\ 025$) (Fig 2) The plasminogen content in the Arthus tonsillitis group (3 8±3 1 casein units/ml) was somewhat lower than that in the control group (7 2±4.8 casein units/ml) but the difference was not statistically significant (Fig 3) The inhibitory ac

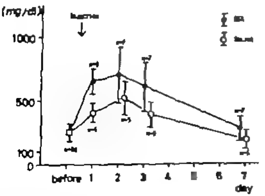

Fig 1 Fibrinogen content after the injection ● the injection of BSA Mean ± standard deviation. O the injection of saline Mean ± standard deviation

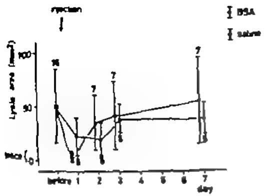

Fig. 2. Whole plasma after the injection ● the injection of BSA, Mean ± S.D. ○ the injection of saline: Mean ± S.D.

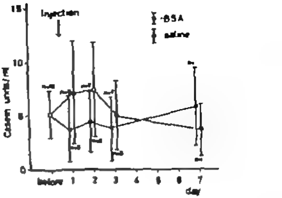

Fig. 3. Plasminogen content after the injection. ● the injection of BSA Mean ± S.D. ○ the injection of saline: Mean ± S.D.

tivity to urokinase of the plasma was almost equal in the Arthus tonsillitis and control groups (Fig. 4).

DISCUSSION

The generalized Schwartzman reaction (GSR) has been used as a model of disseminated intravascular coagulation (DIC). In experimental tonsillitis the Schwartzman reaction was evoked with various biological substances following Ishio's report (1958). However hematological observations were not made even though detailed histopathological observations were carried out. In the experimental GSR, the inflammatory substances were injected intravenously. However in human tonsillitis it has rather rarely been found that inflammatory agents reach the tonsil through the blood stream except in tonsillitis accompanied by sepsis. On the other hand it was significant that the lacunal cavity and lacunal epithelium were found to be the portions involved in the first inflammatory reaction in the onset of human tonsillitis.

Thus the Arthus tonsillitis described in this paper was pathophysiologically more reasonable than the tonsillitis of the GSR from the viewpoint of the pathway of the inflammatory agent. Compared with the tonsillitis of the GSR, no marked necrosis or abscesses were observed in the inflammatory locus in the present experiment. Since it has been shown that the antigen–antibody complex or various proteases from infiltrated leukocytes may be an important factor as cytotoxic agents (Takaba et al. 1964; Thomas 1964) these results might reflect differences in immunoreaction to antigen (BSA). Furthermore in the locus of Arthus tonsillitis the activity of these proteases may be inhibited by protease inhibitors of the blood α_2-macroglobulin and α_1-antitrypsin (Aoki & Moroi 1977; Odani & Ikenaka, 1977). On the other hand marked bleeding and swelling were observed in the Arthus tonsillitis. Such pathological manifestations could be re-

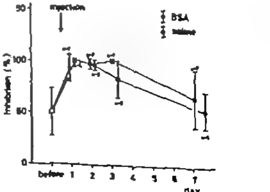

Fig. 4. Inhibitory activity to urokinase after the injection. ● the injection of BSA Mean ± S.E. ○ the injection of saline: Mean ± S.D.

lated to increased levels of plasmin activity On day 1 after the injection the entire plasmin activity was decreased and at this time marked bleeding was observed histopathologically In other words the plasmin in the blood was consumed and at a time corresponding to this bleeding and swelling were apparent

Thrombus formation was not observed in any portion of the tonsil as a result of the thrombolytic condition or thrombolysis due to plasmin In this connection studies were made to determine whether or not the antifibrinolytic agent could inhibit the thrombolysis in the inflammated locus and modify the inflammatory process of the Arthus tonsillitis The mechanism of activation of plasminogen in the circulating blood remained unclear but it has been suggested that plasminogen tissue activator in an inflammatory local site and plasminogen activator from circulating leukocytes may activate plasminogen (Barnhart 1965 Prokopowicz 1968 Henderson et al 1972 Saba et al 1975 Kosugi & Hamaya 1978) Compared with acute tonsillitis in man the changes in the fibrinolytic activity in the blood of rabbits with Arthus tonsillitis resembled the changes following the onset of tonsillitis It is considered therefore that the present Arthus tonsillitis in the rabbit provides a useful model for clarifying the mechanism of the increased fibrinolytic activity observed in acute tonsillitis in man

ZUSAMMENFASSUNG

Die Hypersensitivität der Kaninchentonsillen nach Arthus-Phänomen wurde experimentell untersucht Dabei wurde die histologische Untersuchung der Tonsillen und die Messung der fibrinolytischen Aktivität im Blutplasma durchgeführt Die makroskopischen Befunde waren submuköse Blutung und ödematöse Anschwellung der Tonsillen In den frühzeitigen histologischen Untersuchungen wurden Blutung und Leukozytinfiltration in den verschiedenen Teilen des Parenchyms und Umgebungsbindegewebe nachgewiesen. Auf Grund einer Untersuchung von einigen Parametern des fibrinolytischen Systems im Blutplasma wurde folgendes klar In der frühzeitigen Tonsillitis nach Arthus-Phänomen ist die fibrinolytische Aktivität gesteigert und das gesamte Plasmin (SK-Plasmin) verbraucht Als Ergebnis obiger Tatsachen kommen wir also zu folgender Schlußfolgerung Die Veränderungen der fibrinolytischen Aktivität bei der Tonsillitis nach Arthus-Phänomen im Kaninchen sind fast gleich wie die akute Tonsillitis im Menschen

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T. Kosugi, M.D.
Department of Physiology
Miyazaki Medical College
5200 Kihara Kiyotake-cho
Miyazaki-gun, Miyazaki-ken 889-16
Japan

lated to increased levels of plasmin activity On day 1 after the injection the entire plasmin activity was decreased and at this time marked bleeding was observed histopathologically In other words the plasmin in the blood was consumed and at a time corresponding to this bleeding and swelling were apparent

Thrombus formation was not observed in any portion of the tonsil as a result of the thrombolytic condition or thrombolysis due to plasmin In this connection studies were made to determine whether or not the anti fibrinolytic agent could inhibit the thrombolysis in the inflammated locus and modify the inflammatory process of the Arthus tonsillitis The mechanism of activation of plasminogen in the circulating blood remained unclear but it has been suggested that plasminogen tissue activator in an inflammatory local site and plasminogen activator from circulating leukocytes may activate plasminogen (Barnhart 1965 Prokopowicz 1968 Henderson et al 1972 Saba et al 1975 Kosugi & Hamaya 1978) Compared with acute tonsillitis in man the changes in the fibrinolytic activity in the blood of rabbits with Arthus tonsillitis resembled the changes following the onset of tonsillitis It is considered therefore that the present Arthus tonsillitis in the rabbit provides a useful model for clarifying the mechanism of the increased fibrinolytic activity observed in acute tonsillitis in man

ZUSAMMENFASSUNG

Die Hypersensitivität der Kaninchentonsille nach Arthus-Phänomen wurde experimentell untersucht. Dabei wurde die histologische Untersuchung der Tonsillen und die Messung der fibrinolytischen Aktivität im Blutplasma durchgeführt Die makroskopischen Befunde waren submuköse Blutung und ödematöse Anschwellung der Tonsillen. In den frühzeitigen histologischen Untersuchungen wurden Blutung und Leukozyt infiltration in den verschiedenen Teilen des Parenchyms und Umgebungsbindegewebe nachgewiesen Auf Grund einer Untersuchung von einigen Parametern des fibrinolytischen Systems im Blutplasma wurde folgendes klar In der frühzeitigen Tonsillitis nach Arthus Phänomen ist die fibrinolytische Aktivität gesteigert und das gesamte Plasmin (SK-Plasmin) ist verbraucht Als Ergebnis obiger Tatsachen kommen wir also zu folgender Schlußfolgerung Die Veränderungen der fibrinolytischen Aktivität bei der Tonsillitis nach Arthus-Phänomen im Kaninchen sind fast gleich wie die akute Tonsillitis im Menschen.

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T Kosugi, M.D
Department of Physiology
Miyazaki Medical College
5200 Kihara Kiyotake-cho
Miyazaki-gun, Miyazaki-ken 889-16
Japan

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THE PROGNOSTIC VALUE OF EUSTACHIAN TUBE FUNCTION MEASUREMENTS IN TYMPANOPLASTIC SURGERY

H Virtanen T Palva and T Jauhiainen

From the Department of Otolaryngology University of Helsinki, Finland

(Received December 17 1979)

Abstract The Eustachian tube function was tested preoperatively by sonotubometry and by the negative pressure equalization test in 87 ears subjected to simple myringoplasty tympanoplasty alone or tympanoplasty combined with mastoid surgery The relations between tubal function test results and healing were analysed on average [illegible] months after the operation The results indicating absent or partial pressure equalization ability were poorly correlated with the successful outcome of surgery while sonotubometry showed good correlation Pressure equalization may produce such an unphysiological underpressure that many test results are erroneously negative Sonotubometry is an entirely physiological method and has in this Department replaced the pressure equalization test for routine evaluation of the Eustachian tube function

The prognostic information provided by preoperative Eustachian tube function tests often correlates poorly with the results obtained by tympanoplastic surgery This has recently been documented both by Andréasson & Harris (1979) and by Bluestone et al (1979) in studies on the modified inflation–deflation test originally described by Flisberg et al According to Cohn et al (1979) who used the same negative pressure equalization test, even a total failure to reduce an induced negative middle ear pressure did not preclude successful healing of the tympanum.

The preliminary results of work on 100 ears with tympanic membrane perforation (Virtanen et al 1980) indicate that in the clinical assessment of tubal function the negative pressure equalization test did not succeed as a test for predicting the operative outcome The aim of this work was to compare the results of sonotubometric measurements and the results obtained by the negative pressure equalization method for testing tubal function before and after myringo- and tympanoplasty

MATERIAL AND METHOD

The material consists of 87 patients with central or posterior marginal tympanic membrane perforations mostly following chronic otitis media or rarely injury to the tympanic membrane and ossicles The ages of the patients ranged from 5 to 74 years

Simple myringoplasty was performed on 44 ears The middle ear was dry in all ears The remaining 43 patients were subjected to myringoplasty combined with ossiculoplasty or mastoidectomy

Tympanic membrane repair was made with temporal muscle fascia by the underlay technique and the swing-door exposure (Palva et al 1969) The ossicular reconstruction was performed with ossicular remnants or homografts by methods described earlier (Palva et al 1973)

Preoperative Eustachian tube function was evaluated by sonotubometry and by the negative middle ear pressure equalization test as described in detail elsewhere (Virtanen 1977 1978 Virtanen et al 1980) Figure 1 shows a diagram of the sonotubometry equipment and testing method The continuous pure tone of 6 7 and 8 kHz was introduced into one nostril and registered at the external ear canal during swallowing. An amplitude change of 5 dB or more was taken to denote tubal opening. In the negative pressure equalization test (aspiration

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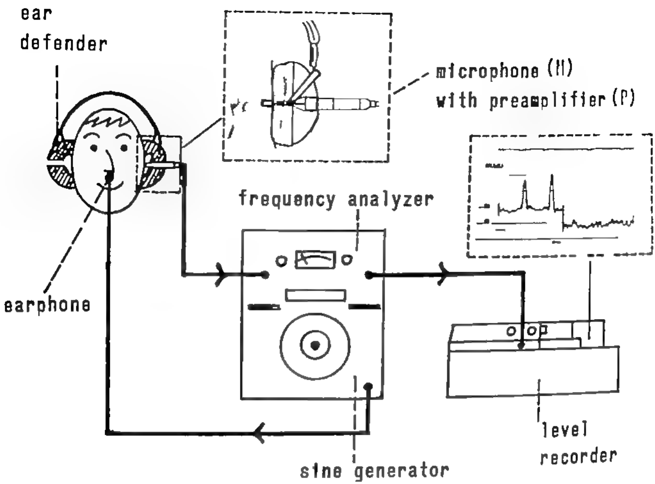

Fig 1 Schematic presentation of the sonotubometry equipment and method For further details see text

method) introduced by Flisberg et al in 1963 and modified by Holmquist (1969) a negative pressure of 200 mmH_2O was produced in the ear canal and the middle ear by means of the air pressure pump of the impedance meter Active equalization of this underpressure was attempted by letting the patient swallow ten times (with a sip of water) The final residual pressure level was registered None of these patients had signs of upper respiratory tract infection at the time of the test Hearing was tested with pure tone audiometry pre and postoperatively and the mean air and bone conduction threshold levels were calculated for the frequencies of 500 1000 and 2000 Hz The mobility of the tympanic membrane was examined with the aid of a Siegle s otoscope and tympanograms were made The shortest postoperative follow-up was 3 months the longest 27 months the average being 13 months The patients were grouped according to the length of the follow-up time 3–4 or 3–5 mo 6 to 11 mo 12 to 23 mo and 24–27 mo respectively (Figs 2 and 3)

RESULTS

Ears subjected to myringoplasty

Among the 44 ears undergoing simple myringoplasty (Fig 2) 11 ears were followed for 3–4 months All had good tubal passage for sound during swallowing prior to surgery In this group the negative middle ear pressure of 200 mmH_2O had remained unchanged during swallowing in 8 ears All 11 ears healed well showing mobile tympanic membranes

The group with 6 months of follow-up includes 16 ears All ears had shown positive sonotubometry results preoperatively The artificially established negative middle ear

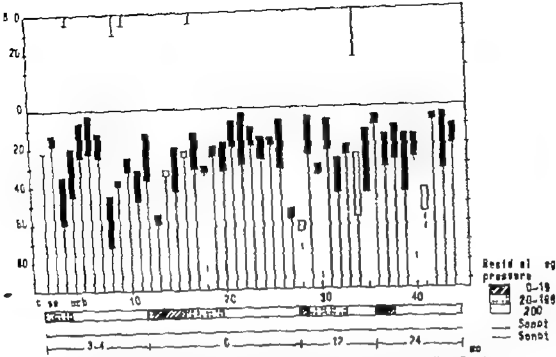

Fig. Individual results with regard to hearing and hearing related to Eustachian tube function measurements in 44 ears undergoing myringoplasty. ■ Hearing improvement. □ hearing loss. Postoperative perforation. The upper part of the graph shows bone conduction in cases in which the loss exceed 10 dB. All values are average values at the frequencies 500, 1000 and 2000 Hz. The area at the bottom of the graph shows the preoperative results of the tubal function tests and the follow-up time. For further details, see text.

pressure of 200 mmH_2O had not been equalized at all in 8 ears, in 4 ears there had been partial and in 4 ears full equalization. In case 18 a small reperforation developed. The negative residual pressure in this ear had preoperatively been −140 mmH_2O and sonotubometric tests were positive both pre- and postoperatively.

Of the remaining 17 ears (cases 28–44) with a follow-up time of 1 to 4 months only one ear (44) had a negative sonotubometry test preoperatively. This patient suffered from allergic rhinitis which at times caused a sensation of blocking in the ears. In this group the pressure equalization test had shown total equalization in 3, partial in 4 and no equalization in 10 ears. In this group with the longest follow-up the sonotubometric measurements were also made postoperatively and the test was positive for all ears.

Postoperatively dry perforation developed during the first 3 months in five ears. In one ear (case 28) complete equalization of the negative pressure had been observed. In one ear (case 30) the residual pressure had been −90 mmH_2O and in two ears (cases 33 and 40) the negative middle ear pressure had remained unchanged during swallowing. The fifth ear (41) had had an infected perforation prior to surgery and no equalization had been noted preoperatively.

Examination of these data on simple myringoplasty reveals that a successful end result in terms of an intact, mobile tympanic membrane and a well-aerated tympanum was achieved in 38 out of 44 ears (86%). Only one patient had had a negative sonotubometric test result preoperatively. Twenty-six of these 44 ears (59%) had preoperatively been totally unable to equalize the negative pressure of 200 mmH O introduced into the ear canal and middle ear but nonetheless 23 were well ventilated post

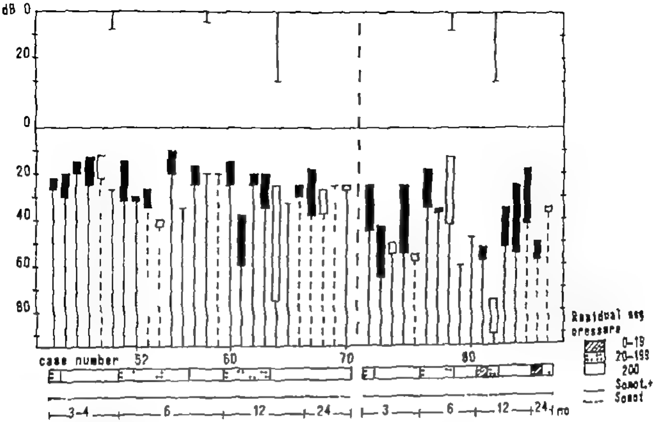

Fig 3 Individual results in 43 ears subjected to myringoplasty or tympanoplasty combined with mastoidectomy For further details see text

operatively Three ears with postoperative perforation had not been able to equalize the middle ear pressure 2 had shown partial and one complete equalization In all 6 ears in which the repair of the tympanic membrane was unsuccessful preoperative sonotubometry had given positive results

Ears undergoing myringoplasty combined with simple mastoidectomy

This group includes 26 ears representing cases 45 to 70 in Fig 3 All ears had been discharging intermittently and 5 ears (cases 53 61 and 67 to 69) were actually moist at surgery For 5 patients sonotubometric measurements were negative preoperatively while the artificially established negative middle ear pressure of 200 mmH_2O remained unchanged in 15 ears In 11 ears partial reduction in the negative pressure was observed None could achieve complete equalization

In 14 ears the fascial graft tympanic membrane healed and was mobile postoperatively In these well-aerated ears the sound had propagated well through the tube during swallowing in the preoperative testing. Eight ears in this group had preoperatively been unable to equalize any of the negative pressure of 200 mmH_2O and 6 had done so partially

Two patients cases 46 and 56 had poorly mobile and somewhat thickened tympanic membranes The former had preoperatively been unable to equalize any of the negative pressure of 200 mmH_2O and in the latter ear a residual pressure of -190 mmH_2O had been recorded In both ears sonotubometry revealed no opening response before operation. Two ears (52 and 70) showed adhesive changes at the follow-up examination The former had had a residual pressure of -60 mmH_2O and a positive result on sonotubometric examination while the latter had shown no equalization of the pressure of -200 mmH_2O and a negative result in the preoperative sonotubometric test

In 8 ears a perforation developed postoper

atively (among them were 4 of the preoperatively moist ears). In 2 patients in this group (66 and 69) preoperative sonotubometry yielded negative results. In two ears (53, 54) the residual negative pressure had been −140 and −170 mmH$_2$O respectively, and for all the others no equalization had been recorded. Three patients were studied by sonotubometry postoperatively and all had good tubal passage for sound. One patient (64) had a healed tympanic membrane but surgery caused severe damage to the cochlea.

Ears undergoing tympanoplasty alone or tympanoplasty combined with mastoidectomy

This group comprises 17 ears (cases 71–87). All ears were dry at surgery. Only three ears had yielded negative sonotubometry results preoperatively. Two ears (81 and 86) had shown total equalization of the negative middle ear pressure preoperatively. In 6 ears partial equalization had been recorded and in 9 ears no equalization at all.

In ten ears, the mobility of the repaired drum was good and the tympanum well aerated postoperatively. Three ears (72, 78 and 80) showed adhesive changes at the follow-up examination. Preoperatively two of these (72, 78) had had positive results on testing by sonotubometry. The former had also shown no equalization and the latter partial equalization of the −200 mmH$_2$O pressure. In the third ear (case 80) the results of both the sonotubometry and the pressure equalization tests had been negative.

In four ears, perforations developed postoperatively. All four had had positive tubal responses when studied preoperatively by sonotubometry. Two ears had shown no equalization of the −200 mmH$_2$O pressure while one (86) had shown total equalization and one ear (87) partial equalization (to −60 mmH$_2$O). In case 8_ no repair of the ossicular chain was performed because of a rigid foot plate.

Hearing gain

Examination of the mean figures for air conduction (Figs. 2 and 3) reveals that a satisfactory postoperative hearing improvement was achieved in the myringoplasty group (cases 1–44) in the great majority of cases. One ear (34) showed significant loss due to inner ear surgical damage. In the groups of ears subjected to myringoplasty combined with mastoidectomy and/or tympanoplasty (45 to 87) there was similarly one case with inner ear surgical damage and further loss of hearing. One of the four ears presenting with adhesive changes showed a significant further loss (30 dB) while in the other three the hearing levels did not deteriorate.

The tympanometric data gave a good V type response in those ears (ca. 40%) in which the perforation to be repaired was small and the drum free of tympanosclerotic changes. After a large perforation repair, the majority (ca. 60%) of the tympanometric curves either showed a small peak or were flat, irrespective of the fact that the tympanum was well aerated and the drum mobile, using Siegle's speculum.

DISCUSSION

It is well established that good results can be obtained by tympanoplastic surgery even when the negative pressure equalization test suggests that the Eustachian tube is functioning poorly (Siedentop et al. 1968; Holmquist 1968; Ekwall 1970; A. Palva & Kärjä 1970; Bluestone et al. 1979; Andréasson & Harris 1979; Cohn et al. 1979). According to Tos (1974) the pressure equalization test is unsuitable for the evaluation of tubal function. As there are at present only limited data on comparisons of sound transmission by tube and pressure equalization (Virtanen 1977) we have in this study made both these measurements on the day before surgery in order to find out how these tests are related to achieving an aerated tympanum.

In the group of 44 ears on which simple

myringoplasty was performed there was a poor correlation between the negative pressure equalization test and the outcome of tympanoplastic surgery because of 26 ears in which the test showed absent or defective equalization 23 ears (88 %) had a well-aerated tympanum at the follow up examination On the other hand the correlation between the results of sonotubometry and aerated tympanum was good In 37 (97 %) of 38 ears with a well aerated tympanum sonotubometry results were positive preoperatively

In the groups comprising 43 ears subjected to myringoplasty combined with mastoidectomy or tympanoplasty alone or combined with mastoidectomy comparison of corresponding figures revealed a similar discrepancy between sonotubometry and the negative pressure equalization test Twenty seven of 43 ears had a well-aerated tympanum with mobile tympanic membranes postoperatively Fifteen of these 27 ears (55 %) had poor tubal function according to the pressure equalization test Sonotubometry on the other hand suggested a good preoperative function in 24 ears (90 %)

When the results are examined further it appears that tests showing a pressure equalization within normal limits (negative pressure equalized to 0–19 mmH$_2$O) correlated well with sonotubometry results and in these cases an aerated tympanum can confidently be expected The other two categories of equalization test results (residual negative pressure between 20 and 199 or an unchanged pressure of −200 mmH$_2$O) cannot be accorded much importance e g these results do not indicate that an aerated tympanum cannot be achieved Apparently the artificially induced −200 mmH$_2$O pressure in many ears is unphysiological thus rendering the test result erroneously negative A locking phenomenon may occur an obstruction which the act of swallowing is not able to overcome (Virtanen 1977 Palva et al 1978) Even many abnormally open tubes can be obstructed during this test (Virtanen 1978)

In ears with normal hearing sonotubometric measurements give positive results in 90 to 95 % of all ears (Virtanen 1977 1978 Murti et al 1979) The present material of cases with simple myringoplasty or tympanoplasty in part combined with mastoidectomy showed a similar trend viz. 78 out of 87 ears (90 %) had a normal opening response for swallowing Thus it remains unsettled whether the negative 10 % represents a normal variant or whether among them are cases of real tubal dysfunction To answer this question will take several years as the number of such ears is so very small in this inactive disease group

Some authors have claimed that tubal function has a tendency to improve postoperatively despite the fact that nothing has been done to the tubal tympanic orifice during surgery (MacKinnon 1970 Andréasson & Harris 1979) In our study the sonotubometric results were positive both pre and postoperatively thus rendering no support for these claims The outcome of surgery on ears with negative preoperative sonotubometry results cannot be elucidated until appreciably larger series of such ears are available

Another point may need consideration From the clinical standpoint it is quite obvious that in dry drum perforations and normal tympanic mucosa elaborate Eustachian tube function tests are a waste of time and it is the faultless surgical technique that primarily determines the result From the theoretical and research point of view these studies are of importance at centres particularly interested in this field

The tympanometric studies are of little help in evaluating tympanic aeration in cases with large tympanic membrane grafts The tympanic pressure changes within +200 to −200 mmH$_2$O are too small to move the new drum as they would move a normal tympanic membrane and clinical examination with Siegle's speculum combined with otomicroscopy and audiometric results should be used to give the right answer of normal aeration of the middle ear

To sum up 63 of 78 ears (80 %) with good

tubal function as measured by preoperative sonotubometry showed a healed tympanic membrane with a well-aerated tympanum at the follow-up examination. In 17 ears (15%) a perforation developed postoperatively. However practically all of these were due to technical failures, the surgeons representing both highly experienced and less experienced members of the Department. Our conclusion is that sonotubometric results in the evaluation of the Eustachian tube function are much more in concordance with the surgical successful result than are the results of the pressure equalization test. The new apparatus constructed for sonotubometric measurement (Sertsonen et al. 1979) is now used for routine testing and we no longer employ the pressure equalization test for the preoperative evaluation of Eustachian tube function.

ZUSAMMENFASSUNG

Die präoperative Funktion der Ohrtrompete wurde mit Sonotubometrie sowie der negativen Luftdruckausgleichmethode an 87 Ohren untersucht, wo eine einfache Myringoplastik oder Tympanoplastik oder diese Operation kombiniert mit Mastoidektomie ausgeführt wurde. Die Korrelationen zwischen den Funktionstesten der Tube und den Ergebnissen der Operation wurde im Durchschnitt 10 Monate nach der Operation ausgewertet. Das fehlende oder schlechte Luftdruckausgleichvermögen der Tube zeigte geringe Korrelation mit den Ergebnissen, da in den meisten Fällen die Paukenhöhle doch gut entlüftet war. Dagegen war die Korrelation mit der Sonotubometrie ganz gut. Bei dem Luftdruckausgleichsverfahren entwickelt sich möglicherweise ein unphysiologischer negativer Druck, der zu falschen negativen Ergebnissen führt. Die Sonotubometrie ist eine völlig physiologische Methode und hat in unserer Abteilung die Druckausgleichmethode bei Routineuntersuchung der Tubenfunktion ersetzt.

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T Palva
Department of Otolaryngology
University of Helsinki
Haartmanink 4 E
SF-00290 Helsinki
Finland

myringoplasty was performed there was a poor correlation between the negative pressure equalization test and the outcome of tympanoplastic surgery because of 26 ears in which the test showed absent or defective equalization 23 ears (88%) had a well-aerated tympanum at the follow up examination On the other hand the correlation between the results of sonotubometry and aerated tympanum was good In 37 (97%) of 38 ears with a well aerated tympanum sonotubometry results were positive preoperatively

In the groups comprising 43 ears subjected to myringoplasty combined with mastoidectomy or tympanoplasty alone or combined with mastoidectomy comparison of corresponding figures revealed a similar discrepancy between sonotubometry and the negative pressure equalization test Twenty seven of 43 ears had a well aerated tympanum with mobile tympanic membranes postoperatively Fifteen of these 27 ears (55%) had poor tubal function according to the pressure equalization test Sonotubometry on the other hand suggested a good preoperative function in 24 ears (90%)

When the results are examined further it appears that tests showing a pressure equalization within normal limits (negative pressure equalized to 0–19 mmH_2O) correlated well with sonotubometry results and in these cases an aerated tympanum can confidently be expected The other two categories of equalization test results (residual negative pressure between 20 and 199 or an unchanged pressure of -200 mmH_2O) cannot be accorded much importance e g these results do not indicate that an aerated tympanum cannot be achieved Apparently the artificially induced -200 mmH_2O pressure in many ears is unphysiological thus rendering the test result erroneously negative A locking phenomenon may occur an obstruction which the act of swallowing is not able to overcome (Virtanen 1977 Palva et al 1978) Even many abnormally open tubes can be obstructed during this test (Virtanen 1978)

In ears with normal hearing sonotubometric measurements give positive results in 90 to 95% of all ears (Virtanen 1977 1978 Murti et al 1979) The present material of cases with simple myringoplasty or tympanoplasty in part combined with mastoidectomy showed a similar trend viz. 78 out of 87 ears (90%) had a normal opening response for swallowing Thus it remains unsettled whether the negative 10% represents a normal variant or whether among them are cases of real tubal dysfunction To answer this question will take several years as the number of such ears is so very small in this inactive disease group

Some authors have claimed that tubal function has a tendency to improve postoperatively despite the fact that nothing has been done to the tubal tympanic orifice during surgery (MacKinnon 1970 Andréasson & Harris 1979) In our study the sonotubometric results were positive both pre- and postoperatively thus rendering no support for these claims The outcome of surgery on ears with negative preoperative sonotubometry results cannot be elucidated until appreciably larger series of such ears are available

Another point may need consideration From the clinical standpoint it is quite obvious that in dry drum perforations and normal tympanic mucosa elaborate Eustachian tube function tests are a waste of time and it is the faultless surgical technique that primarily determines the result From the theoretical and research point of view these studies are of importance at centres particularly interested in this field

The tympanometric studies are of little help in evaluating tympanic aeration in cases with large tympanic membrane grafts The tympanic pressure changes within +200 to -200 mmH_2O are too small to move the new drum as they would move a normal tympanic membrane and clinical examination with Siegle's speculum combined with otomicroscopy and audiometric results should be used to give the right answer of normal aeration of the middle ear

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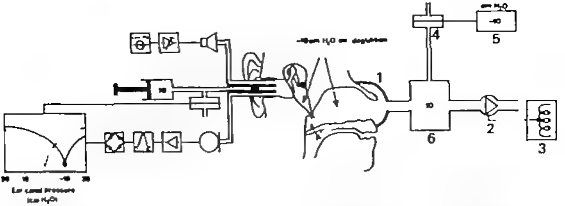

Fig. 1. Equipment for application of relative underpressure in the middle ear cavity from rhinopharynx in association with deglutitions. *1*. Nose mask. *2*. pressure fan. *3*. autotransformer. *4*. pressure transducer. *5*. digital instrument and *6*. pressure equalizer. The middle ear pressure equilibration was controlled by means of a tympanogram recording with the aid of an impedance bridge. ---- Before deglutitions, —— after equilibration of the rhinopharynx pressure.

the pressure generator was pressed airtight over the nose. During swallowing, a suction fan creates an underpressure in the closed rhinopharynx. The suction capacity of the fan is regulated with a variable autotransformer and adjusted by means of a pressure transducer and a digital instrument recording the rhinopharyngial pressure. The pressure generator includes an air container volume 10 litres functioning as a pressure equalizer.

Measurements of the middle ear pressure. The middle ear pressure was estimated from a tympanogram recording (electroacoustic impedance bridge Madsen ZO72) where the pressure in the external ear canal was varied between +20 and −20 cmH_2O (see Fig. 1).

Measurements of the hearing threshold. A Békésy audiometer type Demlar 170 and TDH-49 earphones with MX-41/AR cushions were used. The audiometer was used together with a sound-proof booth. The calibration of the equipment was performed according to ISO R389-1964. The hearing thresholds were recorded at fixed frequencies of 0.5, 1, 2, 3, 4

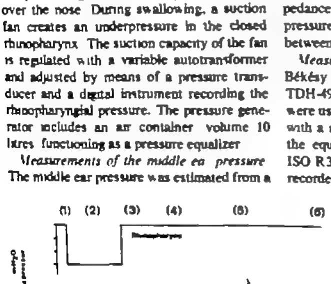

Fig. 2. Recording example in different sequences (*1–6*) for measuring of hearing threshold shift (*HTS*) at relative middle ear pressure −15 cmH_2O and test frequency 0.5 kHz. ---- An early recording of the middle ear pressure by tympanometry during equilibration of rhinopharynx pressure. *Arrow* = Deglutitions.

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THE EFFECT OF STATIC MIDDLE EAR PRESSURES ON THE HEARING THRESHOLD

B Erlandsson [1] H Håkanson [1] A Ivarsson[2] and P Nilsson[2]

From the Department of Physics University of Lund Lund and Department of Otolaryngology University of Lund Malmö General Hospital Malmö Sweden

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Abstract The effect of static tympanic pressure gradients on hearing sensitivity was studied by introducing relative underpressure of 5 10 and 15 cmH O in the middle ear cavity of six normal ears. A self-recording Békésy audiometer was used to measure the hearing threshold shifts during middle ear pressure equilibrations The threshold loss was most prominent for 0.5 and 1 kHz and less for 4 kHz. A threshold gain was shown for 2 and 6 kHz. Over all test frequencies the threshold shifts were increased with higher relative underpressure in the middle ear cavity In order to evaluate the hearing impairment caused by noise it is therefore important to control the middle ear pressure before hearing is tested A small change in middle ear pressure can be ignored when using the summed hearing thresholds between 2 and 8 kHz.

Ever since the late 19th century numerous investigators have studied the function of the Eustachian tube and the effects of pressure disequilibrium in the middle ear on sound transmission and hearing sensitivity The gas contained by the middle ear is continuously absorbed and this will contribute to an under pressure should the tube become occluded (Ingelstedt & Jonson 1967 Elner 1971) The middle ear pressure has been studied in normal ears and has been compared with the tubal function (Elner et al 1971*b*) When tubal function is perfect the middle ear pressure will remain at the ambient pressure level while in normal ears with impaired tubal function the middle ear pressure is more varied

Since the publication of Gelle s classical test in 1881 a number of studies have been devoted to the effect of static ear canal pressures on hearing sensitivity Békésy (1929) reported that positive and negative pressures of 10 cmH_2O in the external ear canal reduce the subjective loudness of low tones by as much as about 1 5 kHz Higher tones are not greatly affected by such pressure variations (Békésy & Rosenblith 1951) Some investigators (Arnold & Schindler 1963 Truswell et al 1979) have observed that these pressure variations produce threshold losses up to 4 kHz but most prominently for the region between 0.5 and 1 kHz.

There are two ways of applying pressure gradients across the tympanic membrane namely via the external ear canal and via the middle ear cavity A positive pressure in the external ear canal is similar to a negative pressure in the middle cavity but the net result is not exactly the same

This work is part of a series of papers on the accuracy of measuring the hearing threshold as a method for detecting hearing impairment caused by exposure to noise The aims of the present investigation were to establish the effect of different middle ear pressures on the hearing threshold of normal ears and to determine whether the hearing thresholds between 2 and 8 kHz can be measured more precisely if the middle ear pressure is controlled

METHODS

Application of the middle ear pressure In order to apply a constant relative underpressure to the rhinopharynx the apparatus shown in Fig 1 was used A nose mask connected to

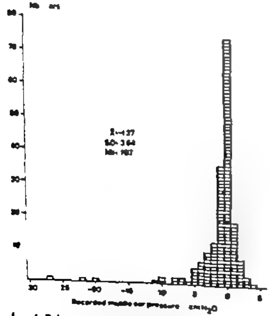
Table I *Hearing threshold shifts in decibels at relative underpressures in the middle ear recorded three times in the same ear*

Middle ear pressure (cmH$_2$O)	Measured hearing threshold shift in dB at different frequencies in kHz					
	Examination	0.5	1		4	6
5	1st	[illegible]	2.1	−2.3	1.0	−0.3
	2nd	4.5	−0.5	0.[illegible]	[illegible]	−0.1
	3rd	4	1.4	−3.[illegible]	3.9	−1.2
10	1st	5.[illegible]	4.9	− 9	0.6	−1.5
	2nd	6.6	9	−[illegible].4	6.3	−1.4
	3rd	7.0	4.8	−8.0	0.1	0.3
15	1st	8.2	3.7	−11.6	1.3	4
	2nd	7.0	1.0	−10.5	7.6	−1.1
	3rd	7.2	4.3	−10.5	4.0	−1.4

just failed to hear the tone and the lower peaks where the subject could just hear the tone (Robinson 1960). We used two linear regressions for calculating the shift of the hearing threshold during middle ear pressure equilibration (see Fig. 2). As the Békésy recording showed an improvement in the mean hearing level just after the recording had started and after the equilibration, we have excluded from our calculations the first 0.5 minutes of the Békésy recording at the beginning of each moment.

B. *Middle ear pressure of workers exposed to noise*

In 95 shipyard workers (187 ears) the initial middle ear pressure was determined from a tympanogram immediately before the hearing tests done during January to March 1979.



F 4 Relat... middle ear pressure of workers exposed to noise immediately before hearing test

RESULTS

A. *Effect of middle ear pressure on hearing threshold*

The threshold shifts in decibels produced by the relative underpressure in the middle ear were recorded for 6 ears at frequencies of 0.5, 1, 2, 4 and 6 kHz. The data are presented in Fig. 3, where positive values denote threshold losses and negative values threshold gains. Threshold losses were recorded at 0.5, 1 and 4 kHz and threshold gains at 2 and 6 kHz. It is also evident from the figure that the gains and losses increase when the relative underpressure in the middle ear cavity is increased.

There is always some uncertainty in the determination of the hearing threshold (Erlandsson et al., 1979*a*) and also the pressure equilibration capacity cannot always be controlled during the test procedure. Therefore the hearing threshold shift of one ear was measured three times at intervals of about one

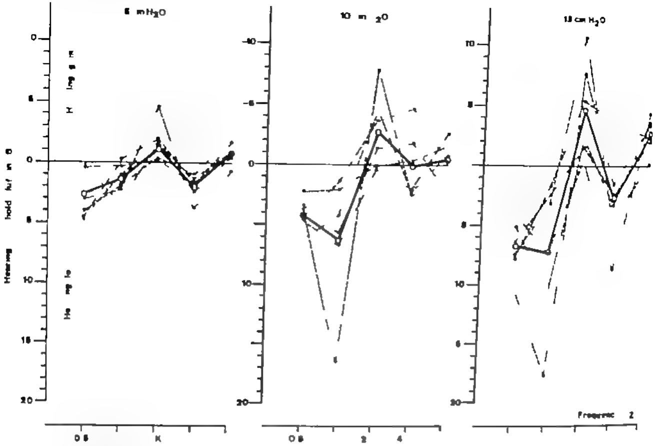


Fig 3 Hearing threshold shifts in decibels at relative underpressures in the middle ear recorded in six normal ears. —— Mean hearing threshold shifts

and 6 kHz The attenuation rate was 2.5 dB $\times S^{-1}$ and the tones were presented at a repetition rate of 2 5 pulses/s and with a 50% duty cycle

MATERIAL AND PERFORMANCE

A *Effect of middle ear pressure on hearing threshold*

Only such subjects were tested as could completely equilibrate relative over and under pressures up to ± 15 cmH_2O by three deglutitions in a pressure chamber (Elner et al 1971*a*) Of 10 subjects six ears (4 subjects) were selected which could completely equilibrate the middle ear pressure to graded underpressures of respectively 5 10 and 15 cmH_2O in the rhinopharynx by five deglutitions This pressure transmission was controlled with an impedance meter (see Fig 1) An investigation of the effects of relative over pressures in the middle ear could not be performed as the gas leaked out through the Eustachian tube

Recordings of hearing threshold changes at different middle ear pressures (−5 −10 and −15 cmH_2O) were made according to the experimental sequences shown in Fig 2. (1) Pressure change in the rhinopharynx (2) five deglutitions (3) the subject is placed in the sound-proof booth and is told not to equilibrate (4) The Békésy test signal is started at 10 dB HL at a fixed frequency and the subject is instructed to begin to use the switch of the Békésy audiometer (5) After 1 5 minutes of Békésy recording the subject is instructed to perform three deglutitions (6) After 3 minutes the recording is stopped

The hearing threshold before and after pressure equilibration was calculated as the mean value between the upper peaks of the trace which represent the points where the subject

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Middle ear pressure (cmH₂O)	Measured hearing threshold shift in dB at different frequencies in kHz					
	Examination	0.5	1		4	6
5	1st	5.8	1	−2.3	1.0	−0.3
	2nd	4.5	−0.5	0.2	[illegible]2	−0.1
	3rd	4.[illegible]	1.4	−3.2	3.9	−1.[illegible]
10	1st	5.2	4.9	[illegible]9	0.6	−1.5
	2nd	6.6	2.9	−2.4	6.3	−1.4
	3rd	7.0	4.8	8.0	0.1	0.3
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B Middle ear pressure of workers exposed to noise

In 95 shipyard workers (187 ears) the initial middle ear pressure was determined from a tympanogram immediately before the hearing tests done during January to March 1979

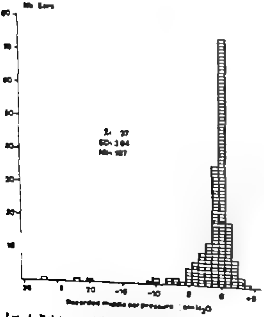

Fig. 4 Relative middle ear pressure of workers exposed to noise immediately before hearing test

RESULTS

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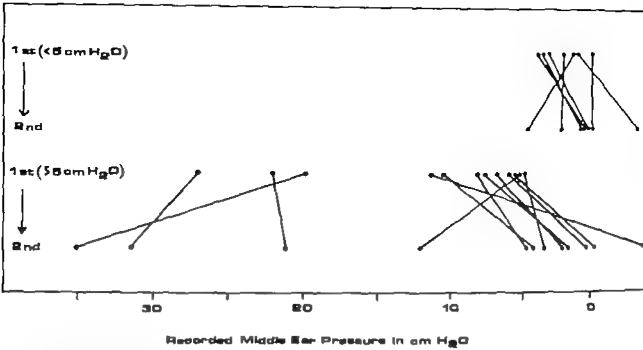


Fig 5 Repeated recording of the middle ear pressure in shipyard workers with relative underpressure <5 cmH O and ≥5 cmH$_2$O at the first recording

week The data are presented in Table I The differences between the values found for the different frequencies were small

B *Middle ear pressure of workers exposed to noise*

The middle ear pressure of 187 ears from shipyard workers was determined indirectly according to the tympanometric method (Fig 4) In most ears (91 %) the measured middle ear pressure was close to the ambient pressure In 16 ears (9 %) the relative underpressure in the middle ear was ≥5 cmH$_2$O Most of these ears were retested after about one month and there was a noteworthy change in the middle ear pressure between the two recordings (Fig 5) The change was not so marked for those who had a relative underpressure (<5 cmH$_2$O)

DISCUSSION

Many factors can affect the variability of the hearing threshold In a retest experiment with the aid of a self recording Békésy audiometer Erlandsson et al (1979*a*) have shown that the standard deviations of the hearing thresholds vary with the test frequencies (Fig 6) On factor may be the variation in the middle ea pressure which was not controlled in this re test experiment The present study shows tha a relative underpressure in the middle ea causes threshold losses at 0 5 1 and 4 kHz an gains at 2 and 6 kHz. In between these point at about 1 5 3 and 5 kHz, the hearing thresh old is not affected by variation in the middl ear pressure This may explain the dip in th standard deviation at 3 kHz shown in Fig 6 This was even more pronounced when th standard deviations of the summed hearin thresholds of 3 and 5 kHz were compared wit the sum of the neighbouring frequencies 2 an 4 kHz The respective values are 4 7 and 6 dB×kHz, underlining the fact that certain fre quencies are affected by the middle ear pres sure and others are not

It is known from earlier studies that a rela tive overpressure in the external ear canal causes hearing threshold shifts With self recording Békésy audiometry in normal ears Arnolds & Schindler (1962) and Thruswell et al (1979) have shown that a relative overpres sure in the ear canal produces threshold losses

Standard deviations of hearing thresholds at different frequencies										
kHz	[illegible]	0.75		3	2.0	2	3.0	0	[illegible]	0
50 48	2.5	0	2		3		2	3	3	4

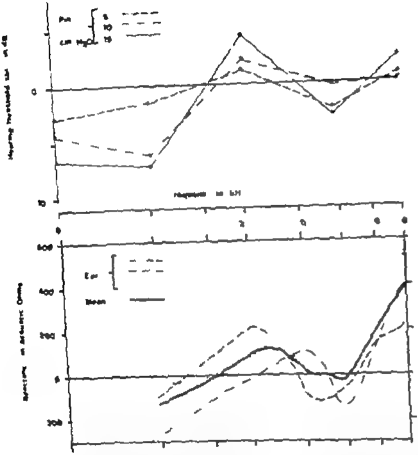
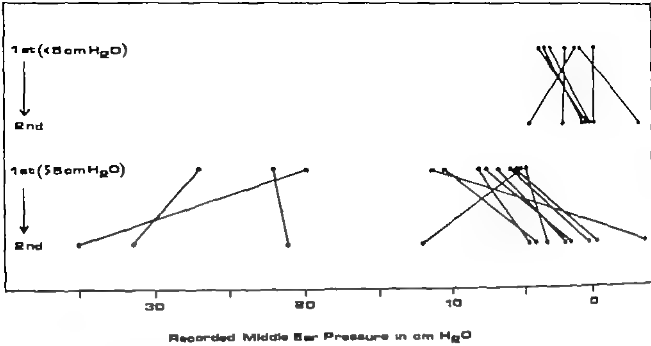


Fig. 6. Upper table shows the precision of Békésy hearing threshold measurements (S.D.) at different frequencies from retest studies of Erlandsson et al. (1979b). Upper diagram shows mean hearing threshold shifts for six ears at different relative underpressures in the middle ear cavity in present studies. Lower diagram shows ear drum reactance of three ears from studies of Mehrgardt & Mellert (1977). —— Mean reactance.

for the frequency range from 0.5 to 1 kHz. These hearing threshold shifts increased with the magnitude of over- and underpressures (see Table II). However, our results for the frequencies 2 and 8 kHz showed a threshold gain.

In the present study we applied pressure gradients across the tympanic membrane from the middle ear cavity, in this way simulating the real effect of a pressure disequilibrium in the middle ear on the hearing sensitivity. Such a middle ear pressure decrease causes an increase in stiffness in both the tympanic membrane and the round and oval windows (Ivarsson & Pedersen, 1977).

The input impedance characteristics of three normal ears seen from the ear drum under normal pressure conditions are shown in



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panic membrane the oval window and the round window Of the approximately 10% of the workers tested the relative underpressure was greater than 5 cmH$_2$O In order to evaluate the hearing impairment caused by noise it is therefore of importance to control the middle ear pressure by tympanometry before testing the hearing A small change in middle ear pressure (<5 cmH$_2$O) relative to the ambient pressure can be ignored by using the summed hearing thresholds between 2 and 8 kHz.

ACKNOWLEDGEMENTS

This work was supported by the Swedish Work Environment Fund grant No. 76/48 We wish to acknowledge our indebtedness to the subjects in this investigation and to Mrs S Bennerup for her enthusiastic work with the experimental measurements

ZUSAMMENFASSUNG

Der Einfluß statischer Druckunterschiede im Mittelohr auf die Gehörempfindlichkeit wurde an sechs Ohren geprüft, indem der relative Luftdruck im Mittelohr 5 10 und 15 cmH$_2$O herabgesetzt wurde Um die Hörschwellenveranderungen während des Druckausgleiches in der Pauke zu messen, kam ein selbstregistrierender Békésy audiometer zur Verwendung Schwellenverluste wurden vorwiegend bei 0 5 und 1 kHz, und in geringerem Ausmaß bei 4 kHz verzeichnet Bei 2 und 6 kHz trat umgekehrt ein Schwellengewinn in Erscheinung Bei samtlichen Prüffrequenzen stiegen die Schwellenveränderungen in direktem Verhältnis zu den Senkungen des relativen Luftdrucks in der Pauke Somit erscheint bei der Beurteilung von Lärmschwerhörigkeit eine Kontrolle des Luftdruckes in der Pauke vor der Hörprüfung als empfehlenswert Geringere Druckveränderungen im Mittelohr können vernachlässigt werden wenn der summierte Hörschwellenwert zwischen 2 und 8 kH ermittelt wird

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Alf Erlandsson M D
ENT Department
General Hospital
S-21401 Malmö
Sweden

Table II *Mean threshold shifts and standard deviations (S D) in decibels produced by static tympanic pressure gradients in the ear canal and the middle ear cavity*

Investigation	Ears n	Tympanic pressure (cmH$_2$O) Middle ear	Ear canal	Frequency in kHz 0 5	1	2	4	6
Present	6	−5		2 9 (2.1)	1.5 (1.3)	−1 0 (2.1)	0 (1.0)	−0.9 (1.0)
		−10		4.[illegible] (1.3)	6 1 (5.5)	− 9 (3.2)	[illegible] (7 9)	−0.5 (1 6)
		−15		6.9 (2.2)	7.2 (6.[illegible])	−4 7 (4 0)	[illegible]7 (4 1)	−2.8 (1.6)
Thuswell et al 1979	22		+10	9 2 (2.9)	5 1 (4.2)	5.2 (4 0)	2 4 (3.5)	−1 1 (6 3)
			+20	12.3 (4 9)	11.3 (3 1)	7.3 (3.9)	5.5 (3.6)	−[illegible]1 (4.6)
			+30	15 [illegible] (3.5)	12 4 (6 0)	8 8 (3 6)	6.8 (4 4)	−[illegible]7 (4 7)
			+40	16.8 (3.3)	14 7 (5 1)	10.5 (4.5)	8 8 (5 0)	− 5 (4 8)
Arnold & Schindler 1962	≈10		+10	≈3	≈2	≈1	≈0	
			+20	≈8	≈6.5	≈4.5	≈3	
			+50	≈13	≈9	≈6	≈2.5	
			−10	≈3.5	≈2.5	≈1.5	≈0	
			−20	≈9 5	≈7	≈4.5	≈ 5	
			−50	≈16	≈1	≈7.5	≈3	

Fig 6 (from a study by Mehrgardt & Mellert 1977) One system corresponds to the ear drum ossicles ligaments and muscles with an apparent resonance at about 1 2 kHz (Békésy 1942) the other to the cochlea oval and round windows A middle ear pressure change increases stiffness of both systems which may explain the hearing threshold gain at 2 and 6 kHz These two frequencies correspond to the two maxima of the positive reactance of the mean input impedance characteristic of the middle ear according to Mehrgardt & Mellert which can probably be reduced at an increased stiffness This study also shows a wide variation of the input impedance of the three investigated normal ears which may explain our high standard deviations of the hearing threshold shift in six normal ears (Fig 3) compared with the corresponding lower standard deviations in the retest of one ear (Table I)

The workers exposed to noise had a rather high frequency (9%) of a relative underpressure (≥5 cmH$_2$O) in the middle ear compared with persons with normal ears (Elner et al 1971*b*) This may be due to an inability to equilibrate the middle ear pressures before the hearing test It is known from the study of Elner et al that when the tubal equilibration capacity is reduced the relative middle ear pressure is more varied As seen from the results of the present study when ears with high relative underpressure were retested there were large differences in the middle ear pressures between the two recordings

In a study of the accuracy of hearing measurement as a method for early detection of hearing impairment caused by exposure to noise Erlandsson et al (1979*b*) have shown that the precision of Békésy hearing theshold measurements can be increased by using the summed hearing thresholds between 2 and 8 kHz, i e the variance of the 2 to 8 kHz average is smaller than the variance for each frequency and in this way even a small change in the middle ear pressure causing both threshold losses and threshold gains in the frequency range of 2 to 6 kHz may be of importance

CONCLUSIONS

The results of this study show that a relative underpressure in the middle ear cavity has an effect on the hearing threshold with both positive (hearing loss) and negative (hearing gain) threshold shifts for frequencies in the range from 0 5 to 6 kHz. These results may be explained by the fact that a relative middle ear pressure increases the stiffness of the tym

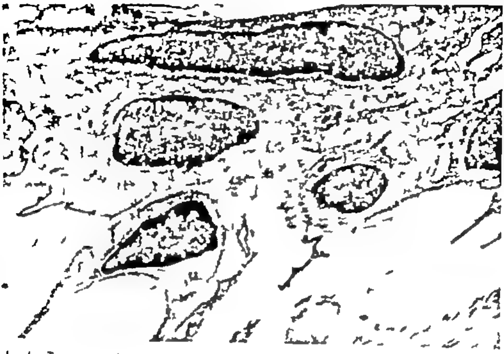


Fig. 1. Transmission electron micrograph (TEM) of normal cat tympanic membrane showing the outer epithelial layer two-three [illegible] thick. TEM × 5 850

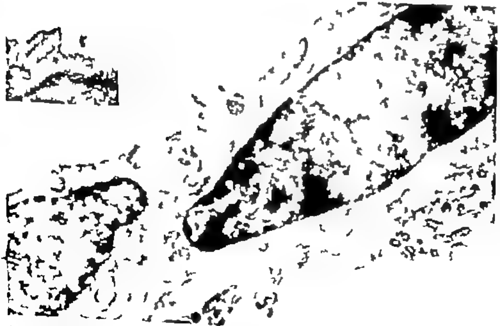


Fig. 1b. The middle ear surface of the membrane is covered by a single layer of mucosal cells with microvilli on the surface. Sometimes ciliated cells (arrow) could be found within this cell layer. TEM × 1 800

Acta Otolaryngol 90 332–341 1980

REPAIR OF EXPERIMENTAL TYMPANIC MEMBRANE PERFORATIONS

L E Stenfors B Carlsöö H Salén and B Winblad

From the Departments of Otolaryngology Histology and Pathology University of Umeå Umeå Sweden

(Received November 19 1979)

Abstract In this investigation the healing pattern of experimental central and marginal pars tensa perforations in cats and rats was studied by using light and scanning electronmicroscopic techniques. The perforations were closed by hyperplastic squamous epithelium exhibiting a marked keratin production. This keratin formed a membranous structure which primarily appeared to close the perforation No ingrowth of squamous epithelium into the middle ear cavity could be detected Generally speaking the perforations had closed within 9–11 days in rats and within 12–14 days in cats.

It is well known to most ENT clinicians that a perforation of the tympanic membrane such as caused by an acute otitis media or an accidental trauma heals spontaneously in the majority of cases In many laboratory animals too experimental tympanic membrane perforations heal within a couple of days

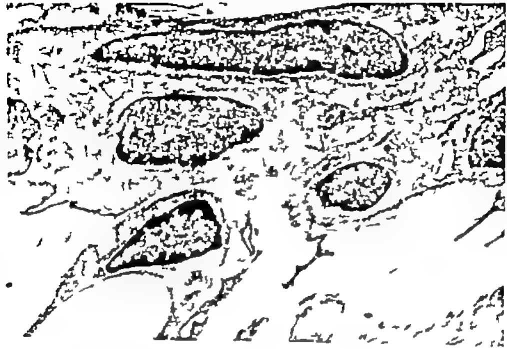
However opinions appear to differ about the cytological healing pattern of tympanic membrane perforations For instance some authors (McInture & Benitez 1970 Clawson & Litton 1971 Reijnen & Kuijpers 1971) advocate a primary epithelial closure of the perforation whereas others (Taylor & McMinn 1965 1967 McMinn & Taylor 1966) have described a primary covering of the defect by granulation tissue followed later on by an epithelial migration Furthermore Dunlap & Schuknecht (1947) proposed that the healing process of drum repair stopped when the hyperplastic squamous epithelium on the external surface has grown over the perforation edge and established contact with the mucosa on the middle ear side of the tympanic membrane

In the present investigation the cytological repair pattern of experimental pars tensa perforations was studied at different time intervals in cats and rats using both light and scanning electronmicroscopic techniques

MATERIAL AND METHODS

A total of 90 tympanic membrane specimens from 16 cats and 29 rats were studied The animals were anaesthetized by an intraperitoneal injection of Ketalar® (Parke Davis) 150 mg/kg (rats) or Mebumal® (ACO Inc Sweden) 30 mg/kg (cats) The animals were all kept under standard laboratory conditions. Any animal showing a sign of external otitis or infection of the middle ear was excluded from the study Under sterile conditions and using an operating microscope central perforations (kidney shaped) of uniform size were made in the pars tensa In 8 tympanic membranes of cats marginal perforations were made by removal of the two posterior quadrants and the adjacent annular ring down to the bony margin In rats the perforations were made through the external auditory meatus and in cats after retro-ventroauricular incision and approach between the concha and the cartilaginous auditory meatus

After various time intervals ranging from 2 days to 10 weeks the animals were re-anaesthetized and the tympanic membranes were carefully examined under the operating microscope After photographing of the membranes the animals were decapitated and the tympanic membrane including the annular ring was removed The specimens were fixed in ice-cold Karnovsky solution (paraformaldehyde/



F 1 Transmission electron micrograph (TEM) of normal ear tympanic membrane showing the outer epithelial layer two-three cells thick TEM 5 850

F 1b The middle ear surface of the membrane is covered by single layer of mucosal cells ith microvilli on the surface Sometimes ciliated cells (*inset*) could be found within this cell layer TEM 12,800.

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Fig. 3. SEM of rat tympanic membrane 7 days after perforation, displaying an almost completely closed defect. SEM ×4

medial surface of the membrane is covered with a single layer of mucosal cells (Fig. 1*b*). The middle layer between the two epithelia consists in the pars tensa of a framework of collagen bundles having typically an outer radial and inner circular arrangement.

The healing pattern is very similar in cats and rats. It varies only slightly between individual animals at different times but in general the repair process follows a similar time schedule.

Gross studies of the perforated membranes in both cats and rats reveal only a slight tendency to closure during the first 2 days though the perforation edges are markedly thickened. After 4–5 days there is a clear tendency toward wound closure and the perforation edge appears opaque and grey in colour. The kidney shaped perforation is now partly filled with detritus and dried secretion (Fig. 2). In the pars flaccida, dilated capillaries are seen and in the attic a thin yellow effusion is noted. This effusion disappears as the perforation heals.

During the next few days the gap between the edges narrows (Fig. 3) and the perforation's border is outlined by a thin, irregular keratin membrane. The perforation is generally completely closed within 9–11 days in rats and within 12–14 days in cats. After 14 days

Fig 2 Scanning electron microscopic (SEM) picture of a rat tympanic membrane viewed from the middle ear side 5 days after perforation The kidney shaped defect is partly filled with detritus and dried secretion SEM ×60

glutaraldehyde 1 1) for 24 hours, rinsed in buffer and transferred to 10% EDTA (cats) Decalcification was generally completed within 10–14 days After decalcification the specimens were dehydrated in graded ethanol solutions and embedded in Epon 812 Semithin (1 μm) sections were cut on an LKB ultratome and stained with toluidine blue The middle ear specimens from 19 rats were primarily decalcified for 24 hours in New Decalc® (manufactured by Histolab Betlehem Trading Gothenburg Sweden) and embedded in paraffin employing routine laboratory techniques The sections were stained with haematoxylin-eosin and according to van Gieson

The middle ear specimens from 10 rats were dehydrated in increasing concentrations of ethanol and ethanol amylacetate up to a concentration of 100% amylacetate and then dried by the critical point drying procedure using CO_2 They were then mounted on blocks and under continuous rotation and tilting covered with an approximately 200 Å thick layer of gold Finally the specimens were inspected using a Cambridge Stereoscan S4 scanning electron microscope (SEM)

RESULTS

The normal tympanic membrane in both animals is covered on the meatal surface by an epithelium two–three cells thick (Fig 1a) The

Fig. 5. Light micrograph (LM) of rat tympanic membrane 48 hours after perforation, showing a 'naked' lamina propria which forms the perforation border (arrow). External auditory canal (*EA*). Middle ear (*ME*). Htx eosin, ×200

At 7–9 days the keratin membrane is observed to bridge over from one wound margin to the other and after contact between the two edges has been established the advancing squamous epithelium appears to close the perforation. The supporting connective tissue can at this stage only be visualized at the periphery and not within this primarily epithelial bridge (Fig. 7).

During the next few days following the perforation closure the squamous epithelium—and later on also the middle layer (lamina propria) of the tympanic membrane—becomes thinner. However, even 10 weeks after the perforation the lamina propria is still clearly thickened and does not exhibit its regular fibre architecture (Fig. 8).

The marginal perforations follow the same healing pattern as do the central ones (Fig. 9). No squamous epithelium can be observed

Fig. 6. LM of cat tympanic membrane 4 days after perforation, showing the perforation edge with hyperplastic squamous epithelium partly covered with keratin spur. Epon-embedded section, toluidine blue, ×220

Fig 4 Photograph of a cat tympanic membrane viewed from the middle ear side 5 weeks after perforation The handle of the malleus protrudes freely into the middle ear The normal adherence of the membrane to the full length of the handle is thus lost and a subsequent flattening of the normally conically shaped membrane is noticed [0]

only one persistent perforation was observed (cat) among all the animals studied of both species The healed tympanic membrane does not exhibit the normal conical shape It is more flattened and only attached to the upper part of the handle of the malleus the unattached part of the manubrium protruding into the middle ear cavity (Fig. 4)

Light microscopy at 48 hours reveals a retraction of the epithelium leaving an uncovered middle layer of connective tissue which at this time forms the edge of the perforation (Fig 5) At 2–4 days a pronounced squamous cell hyperplasia with keratinization is detected around the perforation edges A contact between the outer epithelial cells and the mucosal cells can also be discerned at this time (Fig 6)

Parts of the tympanic membrane distant from the perforation reveals a hyperplastic squamous epithelium and an oedematous middle layer sometimes infiltrated by polymorphonuclear leukocytes Even at this stage (2–4 days) a keratin spur is observed advancing from the hyperplastic epithelium at the wo

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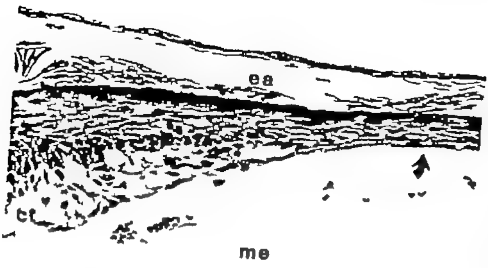


Fig 7 LM 14 days after the perforation (cat) which has been closed by the squamous epithelium (*arrow*) External auditory canal (*EA*) Middle ear (*ME*). Connective tissue in lamina propria (*CT*) Epon-embedded section toluidine blue ×300

growing from the auditory meatus through the perforation into the middle ear cavity at any stage of the healing process

DISCUSSION

The most prominent feature in the initial healing stage of experimental pars tensa perforations in rats and cats is the marked squamous cell hyperplasia observed on the meatal side of the tympanic membrane In a previous investigation (Stenfors & Winblad 1980) concerning the histology of the tympanic membrane after installation of a ventilation tube it was found that even after 7 weeks there was still very prominent squamous cell epithelial

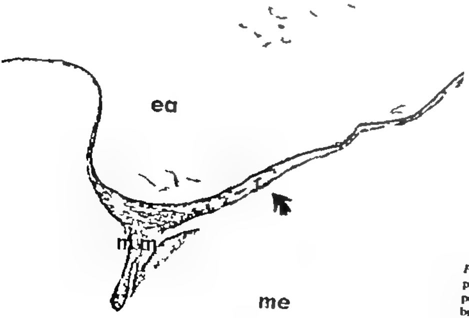


Fig 8 LM 10 weeks after the perforation (rat) The healed portion of the tympanic membrane is thickened (*arrow*) The squamous epithelium displays a normal thickness External auditory canal (*EA*) Middle ear (*ME*) Handle of the malleus (*M*...

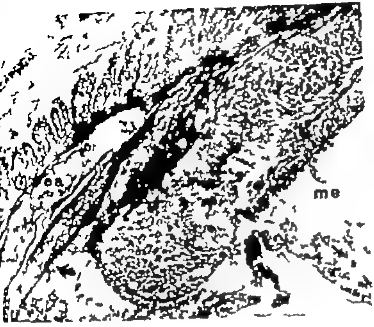
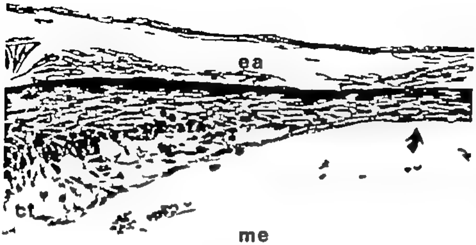


Fig. 9. LM 5 days after marginal perforation (cat), showing hyperplastic squamous epithelium under an advancing keratin spur (arrow). External auditory canal (*EA*). Middle ear (*ME*). Epon-embedded section, toluidine blue. ×220

hyperplasia around the polyethylene grommet. Thus it seems evident that this squamous cell hyperplasia is an essential component in the normal healing mechanism of the tympanic membrane and as long as this hyperplasia persists the membrane has a potential to heal. However, after closure of the perforation the hyperplasia very quickly disappears, whereas even after 10 weeks the lamina propria is still thickened and the normal regular collagen architecture is not yet re-established.

Within 3–4 days the hyperplastic squamous epithelium establishes contact with the mucosal cells on the middle ear side of the drum. According to Stinson (1936) and to Dunlap & Schuknecht (1947) the process of drum repair stops when the stratified squamous epithelium has grown over the perforation edge and approaches the epithelium of the mucous membrane of the middle ear, resulting in a persistent perforation. However, in the present study this contact between the two epithelia was a constant finding at a very early stage of the healing process. Observations similar to ours have also been described by Reijnen & Kuijpers (1971), according to whom this contact situation does not appear to constitute the end of the healing process, as was proposed by Dunlap & Schuknecht (1947).

Habermann (1889) introduced the so-called immigration theory, according to which the auditory meatal squamous epithelium could grow into the middle ear cavity and develop a cholesteatoma. This theory was supported by Rüedi (1948, 1958, 1963), Ojala & Saxén (1951), Friedmann (1955), Kern (1958), Fernández & Lindsay (1960), among others. Our experiments with marginal perforations do not support this theory, however, and we have not been able to find any ingrowth of auditory meatal skin epithelium into the tympanic cavity. Similar negative findings have also been reported by Taylor & McMinn (1965), among others, and thus there would seem to exist, at least in cats and rats, a natural barrier between the squamous epithelium of the external auditory meatus and the mucosa of the middle ear in an otherwise healthy ear. Reeve (1977) noted that keratin production is a prominent feature at all stages in the healing process of the tympanic membrane. Furthermore, Reijnen & Kuijpers (1971) assert that horny



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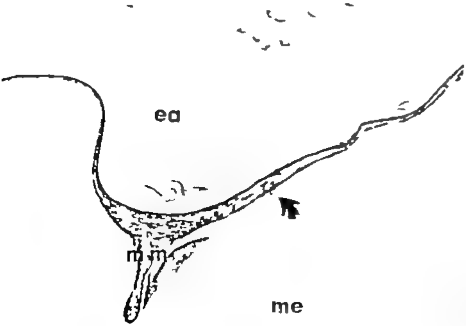

Fig 8 LM III weeks after the perforation (rat) The healed portion of the tympanic membrane is thickened (*arrow*) The squamous epithelium displays a normal thickness External auditory canal (*EA*) Middle ear (*ME*) Handle of the malleus (*MM*) Htx-eosin, ×100

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Dr. Lars-Eric Stenfors
Department of Otolaryngology
University of Umeå
S-901 87 Umeå
Sweden

amellae are propelled in the direction of the regeneration primarily covering the defect In our experiments where specimens are embedded in Epon 812 this keratin spur can be more easily visualized than in paraffin sections It appears that early in the repair process this keratin spur produced by the hyperplastic squamous epithelium primarily covers the perforation The present findings are in agreement with those of Clawson & Litton (1971) and Reijnen & Kuijpers (1971) These authors stated that the primary closure of tympanic membrane perforations is effected by the epithelium followed by mesenchymal cells rather than the converse

To sum up—traumatic central and marginal perforations of the tympanic membrane in cats and rats heal in a three stage process First the hyperplastic squamous epithelium at the perforation edges exhibits an excessive keratin production primarily the perforation gap appears covered by keratin Secondly the perforation is closed by proliferation of the squamous epithelium Finally in the third stage the three layer structure of the tympanic membrane with an outer squamous epithelial layer a middle layer of young connective tissue and an inner mucosal cell layer is re-established Following closure of the perforation the hyperplasia of the squamous epithelium quickly disappears whereas even 10 weeks later the lamina propria is still obviously thickened In rats the perforations are generally closed within 9–11 days and in cats within 12–14 days

ACKNOWLEDGEMENT

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For technical assistance we are indebted to Bengt Carlsson Siw Domeij Per Horstedt, Andrea Jonsson and Ann-Britt Pettersson.

ZUSAMMENFASSUNG

Ziel dieser Untersuchung wurde der Heilungsverlauf von zentralen und randständigen Perforationen der Pars tensa bei Katzen und Ratten im Lichtmikroskop und Rasterelektronenmikroskop studiert. Die Perforationen wurden durch hyperplastisches Plattenepithel mit reichlicher Keratinproduktion überbrückt Dieses Keratin bildete eine membranöse Struktur die primär die Perforation zu schließen schien. Einwuchs von Plattenepithel in die Paukenhöhle konnte nicht entdeckt werden Die Perforationen heilten im allgemeinen bei Ratten in 9–11 Tagen und bei Katzen in 1 –14 Tagen

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The different cellular types can be identified in greater detail by studying their ultrastructural morphology However middle ear effusions have rarely been studied with the electron microscope Therefore to clarify the cellular findings found in light microscopic investigations and the search for possible new ideas concerning the pathogenesis of secretory otitis media, we decided to study the ultrastructure of mucoid middle ear effusions

MATERIAL AND METHODS

Nineteen specimens of mucoid middle ear effusion were taken during tympanostomy from 14 patients 9 males and 5 females aged from 10 months to 12 years All of them had had symptoms of otitis media with effusion of 2–6 months duration. None of the children had any signs of acute infection at the time of tympanostomy nor had they received antibiotic therapy during the last 3 weeks prior to the procedure The specimen taken up into a sterile glass suction tip was immediately dissolved in a tube with 5–10 ml of Ringer's solution and centrifuged at 3000 rpm The supernatant was discarded and the sediment fixed in 3% cacodylate-buffered glutaraldehyde postfixed in osmium tetroxide dehydrated in alcohol and embedded in Epon. Ultrathin sections were made on an LKB Ultrotome III stained with uranyl acetate and lead citrate, and examined with Jeol JEM 100 and T8 electron microscopes.

RESULTS

Variously preserved cellular material was seen in all specimens Neutrophil granulocyte was the most frequently found cell type (Fig. 1) In general they contained phagocytosed material including identifiable cellular organelles e g mucus granules disintegrated debris, and often double membrane-bound structures resembling bacteria (Fig.) The neutrophils showed abundant glycogen granules in 6 and a few in 13 specimens

Macrophages were also present in all specimens (Fig 5) but they were fewer than neutrophils Their sizes ranged from 12–20 μm to 45–54 μm depending on the amount of ingested material Most macrophages contained phagocytosed mucus granules, in some cases whole cells and products of cellular disintegration including myelin figures (Fig 5) A few monocytes (Fig 3) and polyblasts (Fig 4) i e intermediary forms between monocytes and macrophages were observed.

Small and medium sized lymphocytes 8–11 μm in diameter were present in 17 specimens and they were fewer than neutrophils and macrophages (Fig. 6) No plasma cells eosinophil granulocytes or mast cells could be identified in any of our specimens

Large amounts of cellular material in various stages of disintegration were found in all specimens (Fig 7*a* *b* *c*) partly obscuring the origin of these cells However a number of them could be identified either as mucous (Fig. 8) or ciliated epithelial cells (Fig. 9) Others seemed to be inflammatory cells in origin In addition the effusions contained numerous identifiable cytoplasmic organelles, such as mucus granules (Fig. 10*a*) mitochondria (Fig. 10*b*) and lysosomes (Fig. 10*a*) naked nuclei (Fig. 7*a*) and whorled remnants of membranes or so-called myelin figures (Fig. 10*c*)

DISCUSSION

In this material neutrophil granulocytes were the most common inflammatory cells in the mucoid middle ear effusion. This stresses the difficulty in distinguishing mucoid from mucopurulent effusion (Senturia, 1976) The chemotactic agents for neutrophils and macrophages in the middle ear may be bacteria or products of tissue breakdown We found an abundance of the latter in all the effusions while the former were found in a few neutrophils in some of our specimens These agents intensify the emigration of leukocytes from blood ves-

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ULTRASTRUCTURAL MORPHOLOGY OF MUCOID EFFUSION IN SECRETORY OTITIS MEDIA*

P Sipilä,[1] S Sutinen [2] S H Sutinen[2] and P Karma[3]

From the Departments of Otolaryngology and Pathology University of Oulu O lu and the Department of Clinical Sciences University of Tampere Tampere Finland

(Received November 30 1979)

Abstract The ultrastructural morphology of 19 mucoid middle ear effusions in 14 children with secretory otitis media was studied. Phagocytosing neutrophil granulocytes were the most common inflammatory cells and ingested bacteria were present in some of them. The next in frequency were macrophages and lymphocytes Monocytes and polyblasts were also present in most specimens. No plasma cells eosinophil granulocytes or mast cells were seen Epithelial cells were common, and great numbers of free and phagocytosed mucus granules were found Considerable numbers of all the celltypes were in various stages of disintegration Thus it seems that the effusion in secretory otitis media is primarily of inflammatory origin and the dissolution of the cells with liberated cellular contents together with the secretion of the mucosa, contributes to the formation of the effusion.

After the fundamental work of Bryan (1953) cytological methods were introduced in the study of middle ear effusions Ojala & Palva (1955) were the first to apply these methods in secretory otitis media. Senturia et al (1958) classified middle ear effusions as serous purulent mucopurulent and mucoid Clinically and cytologically it may be impossible to distinguish the mucoid from the mucopurulent category (Senturia, 1976) Both of these and the so-called seromucoid category perhaps represent different phase of the pathological process showing a marked secretory component They have all been included in the clinical entity of glue ear or secretory otitis media (Paparella 1976)

Purulent effusion contains predominantly neutrophil granulocytes and smaller numbers of lymphocytes and monocytes (Senturia et al 1958 1960) In mucopurulent effusion macrophages and cellular remnants are most abundant and as the inflammation subsides the effusion becomes mucoid and contains le[ss] cells but large amounts of mucus strand[s] In contrast to some earlier studies (Koc[h] 1947) eosinophil granulocytes have later bee[n] found only exceptionally in the effusion (Se[n]turia et al 1958 Ojala & Palva 1955)

Palva et al (1976) in their light microscop[ic] cellular analysis of the samples from 13[…] middle ears with secretory otitis media, foun[d] that lymphocytes and neutrophil granulocyte[s] predominated and smaller numbers of mono[-] cytes macrophages and cellular remnant[s] appeared in most specimens Eosinophils an[d] mast cells were rare Bacteria were found i[n] one third of the specimens Thus the cyto[-] logical picture suggested an inflammator[y] etiology

Lim et al (1979) confirmed their cytologica[l] findings by electron microscopy and found the neutrophil granulocyte to predominate in both serous and mucoid effusions Its occurrence was in correlation with positive bacteriological findings suggesting bacterial etiology of those cases Eosinophil granulocytes were found only once

The presence of plasma cells in the effusion has not been explained (Palva et al 1976 Lim et al 1979) However in the lamina propria of middle ear mucosa they have been visualized by light and electron microscopy as well as with the immune fluorescence technique in patients with secretory otitis media (Bernstein et al 1972 Lim 1974)

* Supported by grants from the Emil Aaltonen Foundation and the Paulo Foundation Finland.

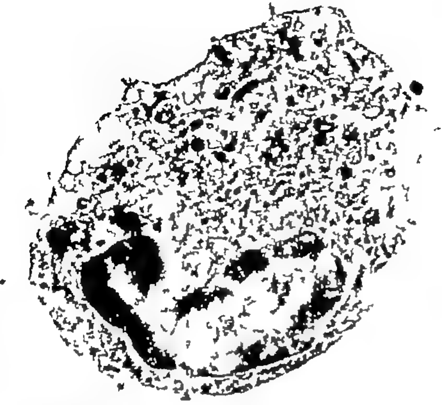


Fig. 3. A monocyte having oval-shaped nucleus and many lysosomes (*L*) in the cytoplasm. ×4 200

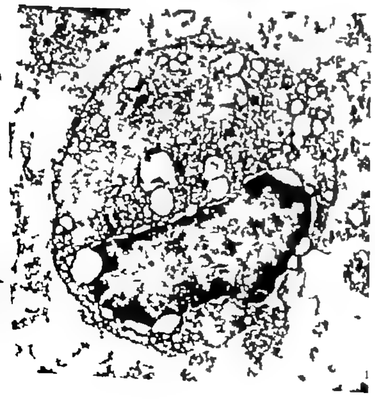
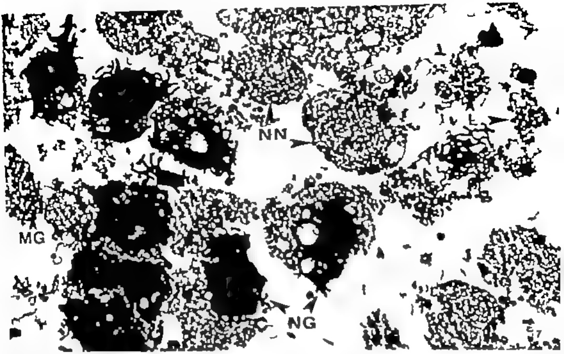


Fig. 4. A polyblast whose nucleus is rounder in shape than the nucleus of the monocyte. Ingested mucus granules (*MG*) can be seen in the cytoplasm. × 500

Fig 1 A polymorphonuclear neutrophil granulocyte with phagocytosed mucus granules (MG) numerous lysosomes (L) and abundant glycogen granules (GG) ×4 200

Fig 2 A greater magnification of the cytoplasm of a neutrophil granulocyte where double membrane-bound structures resembling bacteria (B) and abundant glycogen granules (GG) can be seen ×54 000



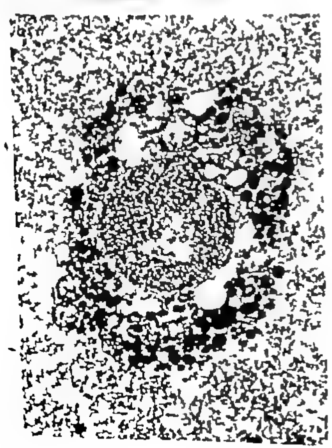


Fig. 7. (a) Disintegrated cellular material. Naked nuclei (*NN*), many free lysosomes (*L*) and mucus granules (*MG*) are visible. Neutrophil granulocytes (*NG*) are phagocytosing disintegrated cellular material. ×1200. (b) Two cells in various stages of disintegration obscuring the origin of the cells. ×4500.

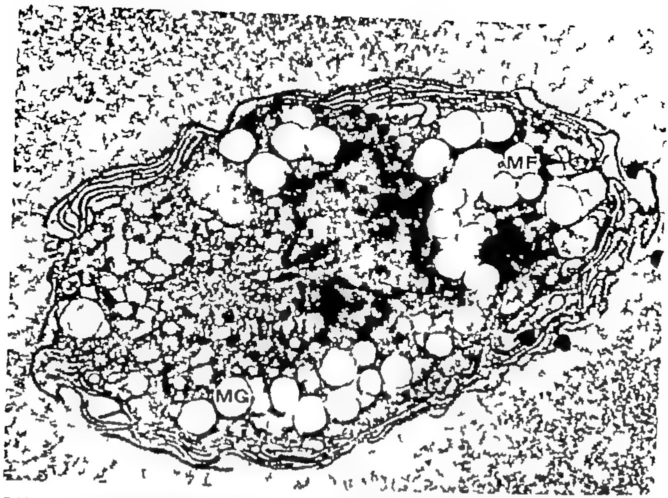


Fig 5 A macrophage with many pseudopods (*P*) for phagocytosis ingested mucus granules (*MG*) and myelin figures (*MF*) ×2 100

Fig 6 A lymphocyte having in the cytoplasm typical Golgi apparatus (*GA*) and mitochondria (*M*) ×4 700

Fig. 9. A ciliated epithelial cell. 5000.

sels and direct them to actual contact with foreign particles which makes phagocytosis possible. Neutrophils move faster than other cell and thus they are usually the first to emigrate. Monocytes are slower in chemotactic motion and lymphocytes have little or no chemotactic activity. Thus the observation supports the idea of a low grade infection as a cause for continued cellular injury in secretory otitis media. Further in two-thirds of our specimens the amount of glycogen in neutrophil was reduced. This might suggest that these cells have used most of their glycogen reserves for energy production in hypoxic environment (Ingelstedt et al. 1975).

The macrophage is an important scavenger cell eliminating bacteria and cellular remnants and it also acts as a carrier of various antigens processing them to T and B lymphocytes (Wing & Remington 1978). In the lamina propria of the middle ear mucosa there is a local macrophage population which may be activated during infection (Lim & Birck, 1971). In addition to macrophages we also observed some monocytes and polyblasts i.e. macrophage precursors (earlier called phagocytes) in the effusion. Thus it seems probable that monocytes are also recruited direct from the blood and activated in the effusion. The activation of macrophages takes place under the influence of an appropriate lymphokine which is the product of T-lymphocytes (Wing & Remington 1978). Our findings support the assumption that a local immune mechanism

Fig. 8. A mucus-secreting epithelial cell having light granules with dark core (*LG*). ×4 100

mediated by the T-cell may exist in the middle ear. Active macrophages acquire phagocytic properties. There is progressive increase in their cell volume and striking enlargement of the Golgi apparatus, which becomes the site of active formation of many small lysosomes. In the same way as in the lung, demand for more macrophages in the middle ear may be met by increased multiplication of free macrophages, release of pre-existing cells from reservoirs within the middle ear, increased production of macrophage precursors, and increased flux of monocytes from the blood to the middle ear (Bram et al. 1978).

The role of the lymphocytes in the pathogenesis of secretory otitis media awaits solution. We found varying numbers of small and medium sized lymphocytes but no plasma cells in the effusion. However, other authors have found abundant plasma cells in the lamina propria of the middle ear mucosa in secretory otitis media (Bernstein et al. 1972, Lim 1974). The products of the plasma cells, i.e. immunoglobulins, have also been frequently isolated from middle ear effusions (Bernstein et al. 1977). Thus, the plasma cells apparently are incapable of permeating the basal lamina of the epithelium, and the immunoglobulins in the effusion either come from the blood or are products of the plasma cells in the lamina propria of the middle ear mucosa. The role of T lymphocytes in this process is unclear as yet. In mucoid effusion, T-cell percentage seems to be decreased (Bernstein et al. 1978, Sipila et al. 1979) even though higher percentages have been demonstrated (Palva et al. 1978). We did not see any eosinophil granulocytes or mast cells in the effusion. This is in accordance with other observations (Palva et al. 1976, Lim et al. 1979). Therefore it seems very unlikely that a hypersensitivity reaction of Type 1 should play a role in the pathogenesis of secretory otitis media.

In secretory otitis media the middle ear mucosa proliferates (Lim & Birck 1971). Its epithelium shows an increase of secreting cells, explaining the local secretory nature of effusion and the abundant appearance of many biologically active agents in the effusion (Lim & Birck 1971). This as well as the abundance of extruded secretory cells and free mucus granules seen in our specimens suggest a true secretory process. Palva et al. (1974) demonstrated that total protein concentration in mucoid middle ear effusion exceeds that in serum. The concentrations of desoxyribonucleic acid (DNA) and many enzymes, such as lactic acid dehydrogenase (LDH), malate dehydrogenase (MDH), acid phosphatase and lysozyme are also higher in middle ear effusion than in serum (Senturia et al. 1958, Gessert et al. 1960, Palva et al. 1974, Juhn et al. 1976). Thus, the above mentioned findings can be well understood in terms of disintegration of cells. We found in the effusion a large number of disintegrating nuclei and lysosomes from both epithelial and inflammatory cells as the source for DNA and lysosomal enzymes.

In conclusion, our findings support the idea that secretory otitis media is an inflammatory disease. It is associated with a dynamic process consisting of an immunological defence system. The effusion is mainly produced by secretion of the middle ear mucous membrane. However, also the dissolution products of the inflammatory and epithelial cells form a remarkable component of the effusion.

ZUSAMMENFASSUNG

Die ultrastrukturelle Morphologie von 19 mucoiden Mittelohr-Ausflüssen bei 14 Kindern mit sekretorischer Otitis media wurde untersucht. Unter den Entzündungszellen waren phagozytierende neutrophile Granulozyten am häufigsten und in einigen von ihnen waren noch eingeschlossene Bakterien zu sehen. Weniger häufig waren Makrophagen und Lymphozyten. Monozyten und Polyblasten wurden in den meisten Proben auch beobachtet, aber keine Plasmazellen, eosinophile Granulozyten oder Mastzellen wurden gefunden. Epithelzellen waren allgemein und eine große Anzahl von freien und phagozytierten mucoiden Granula wurde gefunden. Ein beträchtlicher Anteil von allen diesen Zellen befand sich in verschiedenen Stadien der Auflösung. Es scheint also, daß der Ausfluß bei der sekretorischen Otitis media primär entzündlichen Ursprungs ist und daß zusätzlich zur Sek-

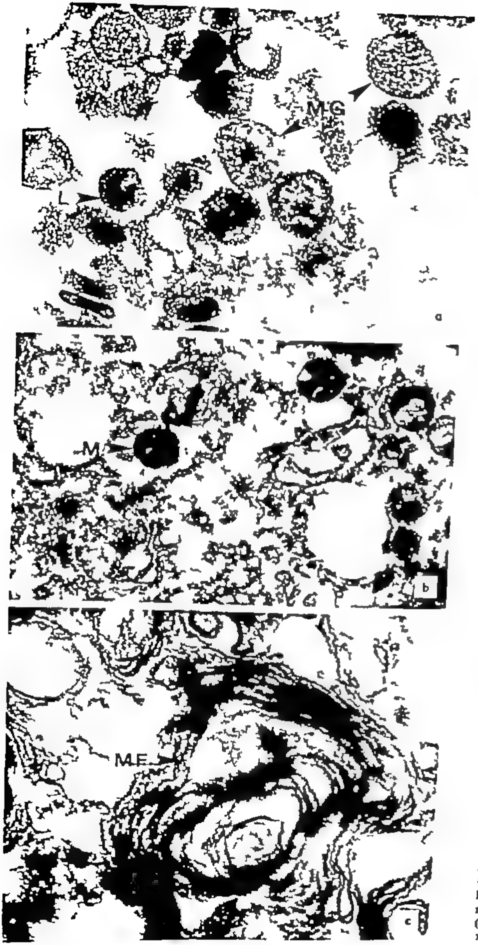


Fig 10 Greater magnification of ( ) mucus granules (*MG*) and lysosomes (*L*) ×13000 (*b*) mitochondria (*M*) ×7000 and ( ) myelin figures (*MF*) × 7000 In the effusion

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THE LOUDNESS OF TINNITUS

Patricia E. Goodwin and Robert M. Johnson

From the Department of Speech Pathology and Audiology, University of Denver, Denver, Colorado, and the Kresge Hearing Research Laboratory, University of Oregon Health Sciences Center, Portland, Oregon, USA

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Abstract. Paradoxically, measurements of the loudness of tinnitus indicate that the noise is not a very loud one, yet many persons experiencing it report severe distress. It has been suggested that either loudness has not been measured correctly or that some other factor such as recruitment may be involved. These aspects were investigated with the following results: (1) the loudness levels obtained for two methods of measuring the loudness of tinnitus differed significantly with the proposed method yielding measures of greater magnitude without exception, (2) recruitment was evidenced for all subjects. On the basis of the results the following conclusions seem justified: (1) the loudness of tinnitus may be more intense than previously reported, (2) the proposed method for measuring the loudness of tinnitus appears to be a more valid measurement than the traditional method.

Tinnitus has been estimated to be a complaint of 32% of the adult population of the United States (U.S. Department of Health, Education and Welfare, 1968). Approximately 20% of these (or 6% of the total adult population) suffer from severe, debilitating tinnitus. Paradoxically, the measurements of the loudness of tinnitus indicate that it is not a very loud noise. Fowler (1941, 1943) reported that the loudness of tinnitus was commonly measured at only 5 to 10 dB SL. Graham (1960) reported that 53.4% of his 73 subjects experienced tinnitus of 5 dB SL or less, 75.3% experienced tinnitus of 10 dB SL or less, 95.8% experienced tinnitus of 20 dB SL or less, and all experienced tinnitus at 30 dB SL or less. Reed (1960) reported that 41% of his 91 subjects experienced tinnitus of 5 dB SL or less, 69% experienced tinnitus of 10 dB SL or less, 87% experienced tinnitus of 20 dB SL or less, 95% experienced tinnitus of 30 dB SL or less, 98% experienced tinnitus of 40 dB SL or less, and all experienced tinnitus of 50 dB SL or less. Atherley et al. (1968) reported that 51% of their 49 subjects experienced noise induced short duration tinnitus (NIST) at a median of 5 dB SL or less, 65% experienced NIST at a median of 8 dB SL or less, 79% experienced NIST at a median of 11 dB SL or less, all experienced NIST at a median of 12 dB SL or less, and the median loudness level for all frequencies was 9 dB SL. Vernon (1976) reported that all of his patients suffered from severe tinnitus but none had ever experienced tinnitus of more than 20 dB SL. Vernon (1978) later reported that in 513 tinnitus patients with severe tinnitus, the loudness of tinnitus was usually measured at 5–10 dB SL; however, he had recorded it at 40 dB SL in some patients and at 70 dB SL in one patient.

Therefore, reports of the measurement of the loudness of tinnitus in the literature indicate that the majority of the tinnitus patients experience tinnitus at a level of within 5 to 10 dB of their thresholds and that almost all tinnitus patients experience tinnitus within 30 to 40 dB of their thresholds.

How can what appears to be such an insignificant sound in terms of loudness cause such profound distress to so many people? Vernon (1976) addressed this perplexing question and offered some possible answers. He felt that recruitment might be the factor responsible for the patient's distress. Recruit-

retion der Mucosa der Untergang von Zellen mit Freisetzung zellulárer Bestandteile zur Bildung des Ausflusses beiträgt

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P Sipilä M.D
Department of Otolaryngology
University of Oulu
Kajaanintie 50
SF 90220 Oulu 22
Finland

using pure tone frequencies generated by the audio oscillator and fed through the audiometer to the earphones. Since the tinnitus had been matched when the subject visited the clinic for a tinnitus evaluation, this frequency was corroborated by bracketing until five equal matches were obtained within ±1% of the frequency. In actuality, consistent pitch matches were usually obtained within ±0.5% of the frequency.

There were three classifications of frequencies used in this experiment. The first one, the tinnitus frequency, was that frequency matched to the pitch of the tinnitus. This tinnitus frequency was associated with a threshold of 25 dB HL or greater for all of the subjects. The second frequency was that frequency which corresponded to a threshold within normal limits (20 dB HL or better) and which was the closest such frequency to the tinnitus frequency. This frequency was termed the normal frequency for the sake of brevity. The third frequency was termed the non-tinnitus pathological frequency. The criteria which governed the choice of this frequency were as follows: (1) It was one of the frequencies produced by the audiometer for which a threshold in dB HL was available for the subject. ( ) The threshold for this frequency was similar to the threshold for the tinnitus frequency. (3) This frequency was at least 750 Hz removed from the tinnitus frequency. The closest frequency to the tinnitus frequency which satisfied the above criteria was chosen and was termed the non-tinnitus, pathological frequency for the sake of brevity.

The loudness of the tinnitus was measured by two different procedures. (1) The first involved the traditional process whereby the tinnitus was matched with an external sound in the ear opposite the ear exhibiting the tinnitus to be measured. This procedure was accomplished by introducing the test tone at the frequency selected in the pitch match of the tinnitus in the ear opposite the ear displaying the tinnitus to be matched in loudness. The threshold of this test tone was determined to the nearest decibel. The number of decibels in 1-dB increments above this threshold required to match the loudness of the tinnitus in the other ear was labeled the 'traditional method'.

Before the test, the experimenter instructed the subject as follows:

> I am going t present   tone in your right/left ear (ear opposite the ear with tinnitus to be measured) and I want you to tell me whether my tone is softer than, louder than or equal to your tinnitus in the left/right ear (tinnitus ear).

The intensity of the test tone was then increased in 1-dB increments from threshold until the subject made an equal loudness judgment. The experimenter then bracketed the area judged to be equal in loudness eliciting 'louder than', 'equal to', and 'softer than' responses until five equal matches had been obtained at the same intensity of the test tone.

(2) A second method of establishing the loudness of the tinnitus was to match the tinnitus with a normal frequency in the same ear exhibiting the tinnitus to be measured. Although this is a more difficult task for the subject to perform, considerable precedent that the task can be performed accurately is available in the literature. The procedure for measuring the loudness of the patient's tinnitus in this manner was labeled the 'proposed method'.

The thresholds for the *tinnitus* frequency and the normal frequency were determined to the nearest decibel. The tinnitus was used as the fixed reference stimulus to be matched.

Before the test, the experimenter instructed the subject as follows:

> I am going to present   tone in your right/left ear (tinnitus ear) and I want you to tell me whether my tone is softer than, louder than, or equal to your tinnitus in the same ear.

The intensity of the variable test tone was then increased in 1-dB increments from threshold until the subject made an equal loudness judgment. The experimenter then bracketed the area judged to be equal in loudness eliciting 'louder than', 'equal to', and 'softer than' responses until five equal matches had been

ment of loudness is a phenomenon which is encountered in ears with cochlear hearing losses and which is characterized by the sensation of loudness growing more rapidly with an increase in intensity than it does in normal ears Therefore sounds that are not uncomfortable to a person with normal hearing may be uncomfortable to a person experiencing recruitment Vernon (1976) suggested that either the method for measuring the loudness of tinnitus is not valid or distress is not a function of loudness or some other factor such as loudness recruitment may be operating or some combination of these statements may be true

For loudness measurements of tinnitus most studies suggest that the most common method has been to measure the loudness of the tinnitus by comparing it to the loudness of a test tone at or near the tinnitus frequency in the opposite ear as advocated by Fowler (1938) who used the alternate binaural loudness balance (ABLB) test to measure the loudness level of tinnitus and to measure loudness recruitment This procedure although reliable may not be valid as it gives the investigator only an indication of the loudness level in the opposite ear If either or both ears are experiencing recruitment then the absolute magnitude of the loudness is not known Vernon (1978) reported that about 90% of his 513 tinnitus patients had hearing losses Of the 513 231 or 45% had audiometric configurations typical of a noise-induced hearing loss Therefore it is likely that many tinnitus patients experience recruitment Furthermore it is also likely that many of these patients display bilaterally symmetrical hearing losses and that they display recruitment bilaterally Therefore the ABLB test for measuring the loudness of tinnitus may not be a valid one for many tinnitus patients

The purpose of this study was to examine the loudness phenomenon related to tinnitus and what effect if any the recruitment phenomenon might have on the magnitude of this psychological attribute

METHOD

The subjects were 9 adult males with tonal tinnitus whose audiometric configurations included at least one frequency within normal limits (20 dB HL or better) so that the monaural loudness balance (MLB) test could be administered Of these 9 subjects 5 had bilateral tinnitus and 4 had unilateral tinnitus Thus the total number of ears afflicted with tinnitus was 14 Informed consent was obtained for all of the subjects after the procedures had been fully explained

All testing took place in a double walled audiometric suite with a double-glazed viewing window between the control room and the examination room (Tracoustics model RS 254B) This room conforms to ANSI (1960) standards for ambient noise The pure tone stimulus generation apparatus consisted of an audio oscillator (Hewlett Packard model 200AB) monitored by a frequency counter (Hewlett Packard models 5300A and 5302A) a clinical audiometer (Maico model MA 24) and matched earphones (Telephonic model TDH-49) mounted in MX 41/AR cushions

The acoustic output of the audiometer was calibrated with a precision sound level meter (Brüel & Kjær type 2113) and a high precision measuring condenser microphone (Brüel & Kjær type 4144) housed in a 6-cc standard coupler (NBS 9A) The potentiometers were set so that the output was within one dB of the ANSI (1969) proposed standard Repeated checks of the calibration during the 6-week duration of the experiment indicated no change in the output of the audiometer equal to or greater than one dB

Prior to the experimental procedure air conduction pure tone thresholds were obtained for frequencies of 250 500 1000 2000 4000 and 8000 Hz for each subject utilizing the method recommended by Byers et al (1978) The results of this testing were used to ascertain if the subject would be retained as an experimental subject

A pitch match of the tinnitus was made

using pure tone frequencies generated by the audio oscillator and fed through the audiometer to the earphones. Since the tinnitus had been matched when the subject visited the clinic for a tinnitus evaluation, this frequency was corroborated by bracketing until five equal matches were obtained within ±1% of the frequency. In actuality, consistent pitch matches were usually obtained within ±0.5% of the frequency.

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I am going to present a tone in your right/left ear (ear opposite the ear with tinnitus to be measured) and I want you to tell me whether my tone is softer than, louder than or equal to your tinnitus in the left/right ear (tinnitus ear).

The intensity of the test tone was then increased in 1-dB increments from threshold until the subject made an equal loudness judgment. The experimenter then bracketed the area judged to be equal in loudness eliciting 'louder than', 'equal to' and 'softer than' responses until five equal matches had been obtained at the same intensity of the test tone.

(2) A second method of establishing the loudness of the tinnitus was to match the tinnitus with a normal frequency in the same ear exhibiting the tinnitus to be measured. Although this is a more difficult task for the subject to perform, considerable precedent that the task can be performed accurately is available in the literature. The procedure for measuring the loudness of the patient's tinnitus in this manner was labeled the 'proposed method'.

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Before the test, the experimenter instructed the subject as follows:

I am going to present a tone in your right/left ear (tinnitus ear) and I want you to tell me whether my tone is softer than, louder than, or equal to your tinnitus in the same ear.

The *intensity* of the variable test tone was then increased in 1-dB increments from threshold until the subject made an equal loudness *judgment*. The experimenter then bracketed the area judged to be equal in loudness eliciting 'louder than', 'equal to' and 'softer than' responses until five equal matches had been

obtained at the same intensity of the variable test tone The two methods were presented to the subjects in counterbalanced order

Since the subjects displayed bilateral sensorineural hearing losses typical of noise induced hearing loss such that normal and abnormal thresholds were present in the same ear the MLB test and not the ABLB test was the appropriate measure of recruitment by a loudness balance procedure For the MLB test the frequency associated with the poorer sensitivity was designated the reference or fixed frequency The closest frequency on the audiometer to the tinnitus frequency which corresponded to a normal threshold for the subject (20 dB HL or better) was designated the variable frequency This is the normal frequency as defined above The reference tone was presented for the first test at 20 dB SL and for the second test at 40 dB SL It was presented alternately with the variable tone (0 5 sec on-time and 0 5 sec off-time for each tone) to the same ear

Before the test the experimenter instructed the subject as follows

I am going to present a high tone and a low tone in your right/left ear (demonstrating the high and low tones) Tell me whether the low tone is softer than louder than or equal to the high tone

The intensity of the variable test tone was then increased in 5-dB increments from threshold until the subject made an equal loudness judgment The experimenter then bracketed the reference tone in 1-dB increments and decrements until five equal matches had been obtained at the same intensity of the variable test tone For the MLB test no recruitment was defined as equal loudness levels within ± 10 dB SL Complete recruitment was defined as equal loudness levels within ± 10 dB HL Partial recruitment was defined as equal loudness levels between these two extremes

RESULTS AND DISCUSSION

The null hypothesis used in this study stated that there would be no significant difference between the sensation levels obtained by the traditional method and the proposed method The results obtained for the two procedures are shown in Table I It is immediately apparent that every sensation level without exception was greater for the proposed method than for the traditional method Across subjects the mean difference was more than three times as great for the proposed method than for the traditional method When this difference was analysed statistically the difference between the two procedures was significant at beyond the 0 001 level of confidence (Table II) It is evident from the results of the analysis of variance that the two methods clearly produced different measures of the loudness of tinnitus These differences can probably be accounted for on the basis of the recruitment phenomenon since all of the subjects in this investigation demonstrated loudness recruitment as measured by the MLB test

In order to establish the magnitude of recruitment through a loudness balance technique one must either have an ear with normal sensitivity or an ear with at least one fre

Table I *Sensation levels obtained for the traditional and proposed methods for measuring the loudness of tinnitus*

	Sensation levels	
Subject	Traditional method	Proposed method
1		8
2	5	17
3	3	10
4	1	10
5	8	3
6	19	42
7	7	29
8	4	19
9	8	70
10	1	19
11	70	50
1	3	30
13	11	4
14	1	24
Sums	93	334
Means	6 64	23 86
Standard deviations	6_5	1_05

Table II *Analysis of variance used to test hypothesis*

Source	SS	df	ms	*F*	*P*
Total	4 469 25	27			
Subjects	2 009 75	13			
Treatment	2 074 32	1	074 32	70 01	<0 001
Error	385 18	13	29.63		

quency within normal limits with which to compare the growth of loudness in the pathological frequency or ear For patients who exhibit bilateral hearing losses where there is neither a normal ear nor a normal frequency loudness balance tests are inappropriate as a measure of recruitment since the ear or frequency used as the control may also be exhibiting recruitment

A very similar situation exists for determining loudness for the patient with tinnitus The traditional method of measuring the loudness of the tinnitus has been to present an external stimulus similar in frequency to the tinnitus in the opposite ear and to adjust the signal until the external stimulus was comparable in loudness to the patient s tinnitus Very often the frequency of the external stimulus corresponds to an area where the hearing sensitivity is depressed and the ear i recruiting In these cases the loudness of the external sound would increase rapidly at suprathreshold levels and the differential in the perceived loudness would be con iderably larger than the magnitude of the difference indicated on the dial

The loudness level registered in this study by means of the traditional method were all 20 dB SL or les These results support the finding of several previous investigators (Fowler 1941 1943 Atherley et al. 1968 Vernon 1976) Graham (1960) reported 95.8% and Reed (1960) reported 87% with loudness levels of 20 dB SL or less Vernon (1978) reported that tinnitu wa usually measured at 5 to 10 dB SL, although he had recorded it at 40 dB SL in some cases and at 70 dB SL in one case

Table III presents a comparison of the results of the measurement of the loudness of tinnitus using the traditional and proposed methods in this study with the results obtained by Graham (1960) and Reed (1960) Note the similarity between the results obtained by the traditional method in this study and the results obtained in the study by Graham (1960)

There are two conditions which were not explored in this study the test tone at a tinnitus frequency in the test ear and the test tone at a frequency with a threshold within normal limits in the ear contralateral to the test ear The proponents of the traditional method for measuring the loudness of tinnitus have argued that it was necessary to introduce the test tone at a tinnitus frequency in the ear contralateral to the tinnitus ear so as to minimize interference or masking. This reasoning is supported by the studies in the masking of tinnitus (Feldmann 1971 Vernon 1977 Vernon et al 1977 Vernon & Schleuning 1978) The proposed method used in this study incorporated test tones at frequencies at least 1 000 Hz removed from the tinnitus frequencies None of the subjects reported any masking of tinnitus as a result of these test tones

The last condition—the usage of a test tone at a normal frequency presented *to the non test ear—has not been reported in the literature* and was not investigated herein *Logically it would be expected that the results ob*tained for the same non-tinnitus frequency at a *normal threshold and presented* to the test ear

Table III *A comparison of the loudness of tinnitus for Graham s (1960) and Reed's (1960) studies ith the results obtained in the present study using two methods*

dB SL	Graham	Reed	Traditional	Proposed
0– 5	53.4%	41%	57%	0%
6–10	1 9%	28%	1%	1%
11–20	20 5%	18%	5%	[illegible]
21–30	4 1%	8%	0%	[illegible]
31–40	0 0%	3%	0%	7%
41–50	0 0%	%	0%	14%

Table IV A comparison of pitch and loudness measurements for 9 test ears of 6 subjects obtained on two different dates separated by intervals of 1–4 months

Subject	Interval	Tinnitus frequency (Hz)		Loudness of tinnitus in dB SL			
				Traditional		Proposed	
		(1)	(2)	(1)	(2)	(1)	(2)
1	4 months	cnt	8 414	cnt	2	cnt	8
2	1 month	6 600	7 545	3	5	1	17
3	1 month	6 600	7 545	3	3	12	10
6	2 months	4 000	4 000	5	19	8	4
7	3 months	10 500	13 215	6	7	1	79
8	4 months	8 000	6 870	4	4	15	19
9	4 months	7 000	6 870	3	8	16	70
12	months	3 600	3 034	3	3	5	30
13	2 months	3 600	3 030	3	11	25	24

cnt could not test because subject did not have tinnitus
Subject experienced beats to a test tone of 6 400 Hz.

and to the non test ear would not differ significantly

The reliability of pitch and loudness measurements during the experimental sessions was remarkable in that the subjects were extremely consistent in their pitch matches and loudness balances Reliability measures over time are very difficult in that many people experience tinnitus which fluctuates in pitch and in loudness Six subjects with 9 test ears had received tinnitus evaluations between one and 4 months prior to their participation in the experiment The pitch and loudness measurements of their tinnitus recorded on the two dates are shown in Table IV Some were remarkably consistent while others were not

The problem of determining the validity of the two methods of measuring loudness is equally perplexing Several investigators have questioned the relationship between distress and the loudness of tinnitus Although part of this problem may result from the method used to measure loudness there is also no way to evaluate objectively the degree of distress that a patient experiences Patients respond to distress in a highly individualistic manner and the magnitude of the loudness measurement does not seem to be related to the severity of the complaint This observation is supported by patients who report that their tinnitus is as loud as a jet aircraft taking off although the loudness of the tinnitus is measured as being only 5–10 dB above their thresholds Others report extreme and unremitting anguish actual pain and some entertain thoughts of suicide in order to rid themselves of the tinnitus This would appear to support the hypothesis that the traditional method of measuring loudness of tinnitus is incorrect

The results obtained using the proposed method appear more realistic in that the loudness levels under this condition are of sufficient intensity to be distressful to the patient For example subject 11 had tinnitus which was measured at 20 dB SL by the traditional method and at 50 dB SL by the proposed method (Table I) For the proposed method the tinnitus was balanced in loudness to a frequency at a normal threshold (2 000 Hz, 10 dB HL) at 60 dB HL which is 50 dB SL or 71 dB SPL This is not an insignificant sound In fact it is as loud as a freight train passing 100 feet away (Peterson & Gross 1977) There fore the proposed method for measuring tinnitus appears to be a more valid procedure than the traditional method because the results obtained with the proposed method are more in line with the reality of the tinnitus patient s

distress and the magnitude of the measure is known because the loudness level of the tinnitus is being compared with a frequency at a normal threshold. Neither method is suitable for measuring the loudness of tinnitus in the patient who has a bilateral hearing loss which encompasses the entire frequency range. Alternative methods will have to be devised to measure the loudness of tinnitus in such cases.

ZUSAMMENFASSUNG

Paradoxerweise ergibt das Messen der Lautstärke von Ohrgeräuschen, daß das Geräusch nicht sehr laut ist, daß jedoch viele Menschen, die es erfahren, großes Unbehagen anzeigen. Es wurde vorgeschlagen, daß entweder die Lautstärke nicht richtig gemessen wurde oder daß es an einem anderen Faktor, z. B. Recruitment liegen könnte. Diese Gesichtspunkte wurden geprüft mit den folgenden Ergebnissen: 1) Die Tonstärke, die sich bei zwei Meßmethoden für die Lautstärke von Ohrgeräuschen ergab, war entschieden anders mit der vorgeschlagenen Methode, die ausnahmslos größere Lautstärken ergab. 2) Recruitment zeigte sich bei allen Patienten. Auf Grund dieser Ergebnisse scheinen die folgenden Schlußfolgerungen gerechtfertigt: 1) Die Lautstärke von Ohrgeräuschen könnte größer sein als bisher angegeben. 2) die vorgeschlagenen Meßmethode für die Lautstärke von Ohrgeräuschen scheint eine richtigere als die traditionelle Methode zu sein.

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Patricia E. Goodwin, PhD
Department of Speech Pathology and Audiology
University of Denver
Denver
Colorado 80208
USA

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HEREDITARY DEAFNESS IN THE CAT

An Electron Microscopic Study of the Spiral Ganglion

H H Elverland and I W S Mair

From the Department of Otorhinolaryngology Regionsykehuset University of Tromsø Norway

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Abstract The spiral ganglion from white cats with hereditary deafness has been studied with the transmission electron microscope and comparisons made with hearing animals at different ages Ganglion cell loss occurs secondary to destruction of the organ of Corti but only after the lapse of several months Prior to neuronal loss the type I ganglion cells lose their myelin sheaths and concurrently develop an increased content of neurofilaments Type I neurons transform into type II through an intermediate type III stage This process of neurofilamentous degeneration occurs slowly and phagocytosis is therefore an inconspicuous feature

The Scheibe type of cochleo-saccular degeneration is frequently found in human hereditary deafness (Lindsay 1973) Identical morphological findings have been reported in temporal-bone studies of the hereditarily deaf white cat (Bosher & Hallpike 1965 Mair 1973) which therefore represents a spontaneously occurring animal model for this type of human labyrinthine disease Progressive degeneration of the epithelial elements of the cochlear duct occurs post natally in the deaf white cat and the time-course of this process has been described in both light and electron microscopical studies of large series of deaf animals (Bosher & Hallpike 1965 Mair 1973 Mair & Elverland 1977)

Although a few studies have indicated a primary neural degeneration in some deaf white cats (Alexander & Tandler 1905 Pujol et al 1977) most investigators have concluded that degeneration of the primary afferent neurons occurs secondary to destruction of the cochlear sensory cells (Howe 1935 Mair 1973) In a quantitative study of 48 deaf ears numerical reduction in the spiral ganglion neuronal population was first significant after the age of 10 months (Mair 1973) The purpose of the present investigation was to study the degeneration of the spiral ganglion cells at the ultrastructural level and is an extension of an earlier interim report (Elverland et al 1977)

MATERIAL AND METHODS

A total of 22 cats have been used in this study their ages ranging from 7 days to about 6 years The hearing status in each ear was assessed by recording the early auditory evoked potentials (Mair et al 1978) Two animals had unilateral deafness and the material consists of 30 deaf and 14 hearing ears In the youngest age group colour coat kittens were used for comparison with young deaf animals. The age coat colour and hearing status together with experiment numbers are given in Fig 1

The 7-day-old kittens which were littermates were decapitated under ketamine hydrochloride anaesthesia and the petrous bones immediately isolated The stapes was extracted and a polyethylene tube inserted through the round window membrane After submersion in 1% OsO_4 in 0 1 M phosphate buffer the fixative was repeatedly aspirated via the cochlear turns from the oval to the round window All other animals were deeply anaesthetized with intraperitoneal Nembutal

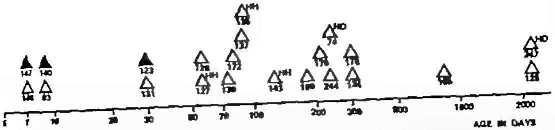

Fig. 1. Age distribution of 19 white coat (Δ) and colour coat (▲) cats. The presence of auditory pathway potentials in white coat animal is designated by *H* whilst *D* indicates the deaf ear in monaurally deaf white-coat cats. All other white-coat animals were bilaterally deaf whilst auditory potentials were present in the colour-coat kittens.

heparinized and then perfused through the abdominal aorta with a fixative consisting of 4% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer. The petrous bones were dissected free and local perfusion of the cochlea with the aldehyde mixture was performed as described above. Following osmification and dehydration in acetone the inner ears were embedded in Spurr and further processed according to the technique of Spoendlin & Brun (1974).

Semithin sections from each coil of the cochlea were stained with paraphenylene diamine to visualize myelin sheaths and studied with the light microscope. Ultrathin sections from representative areas in Rosenthal's canal were examined with a Hitachi HU 11 electron microscope after staining with lead citrate.

RESULTS

In the earliest postnatal period no evidence of pathological changes is found in the spiral ganglion. The ganglion cells in both the deaf white kitten (Fig. 2) and its colour-coat littermate have an identical appearance with a strikingly homogeneous population of ganglion cells. These contain an even distribution of cytoplasmic organelles and are covered by one or two layers of Schwann cell cytoplasm. The maturation of the spiral ganglion proceeds apparently normally in both deaf and hearing animals with development of myelin sheaths.

The first pathological features have been found at the 75-day stage with apparently increased numbers of non-myelinated cells containing numerous neurofilaments (Fig. 3). In successively older animals, it is not unusual to find adjacent ganglion cells in deaf animals which lack myelin sheaths and contain considerable quantities of neurofilaments (Fig. 4). This has never been observed by us in hearing animals. The increased content of perikaryal neurofilaments is associated with partial disappearance of rough endoplasmic reticulum in the peripheral part of the cytoplasm with relative preservation in the perinuclear regions (Fig. 4). Golgi membranes are well preserved while the cytoplasmic content of pigment inclusions and vacuoles is apparently increased. Free ribosomes remain throughout the cytoplasm.

Cells with a considerable perikaryal content of neurofilaments have a single covering layer of Schwann cell cytoplasm (Figs. 3–5) which often separates from the ganglion cell plasma lemma with formation of intervening vacuolar spaces. The intermediate stages are characterized by loose organization of the covering myelin with variable numbers of lamellae. Fragmentation of the myelin lamellae is frequently found together with vacuoles in the Schwann cell cytoplasm and myelin figures (Fig. 6).

Non-myelinated spiral ganglion cells with varying content of neurofilaments present a shrunken appearance and are smaller than the typical type I cells: the nucleus is often found eccentrically located and shows either a

Fig 2 Section from third coil of spiral ganglion from 7 day-old white kitten (no 148) with transitory occurrence of auditory evoked potentials on third postnatal day Note the homogeneous population of ganglion cells and the t sence of myelin sheaths except around axons. Marker 10 μm

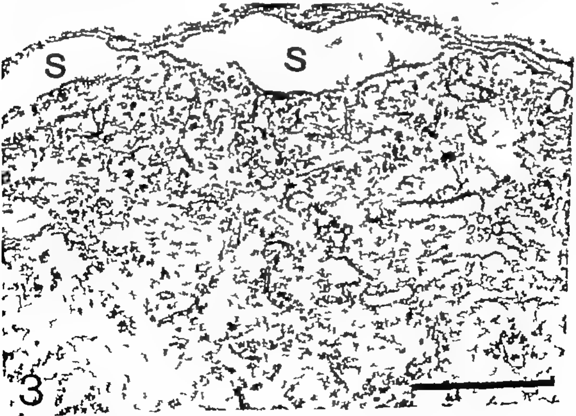

Fig 3 Peripheral part of ganglion cell from pper part of first coil from 75-day-old deaf white cat (no 139) Note the abundant neurofilament content n the cytoplasm Th plasmalemma l covered with one layer of Schwann cell cytoplasm with formation of empty spaces (S) between the perikaryon and the Schwann cell Marker = 1 μm.

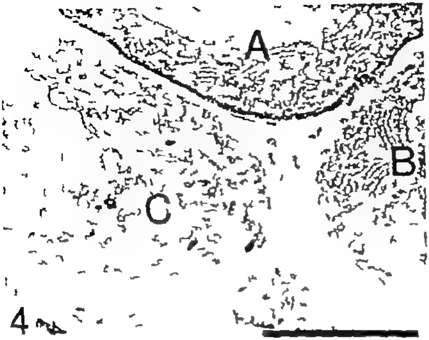

Fig. 4. Ganglion cells from second coil of 10-month-old deaf cat (no. 134). Cells *A* to *C* show an increasing content of neurofilaments with loss of rough endoplasmic reticulum. Cell *A* is myelinated while cells *B* and *C* have only a single Schwann cell layer. Marker=5 μm.

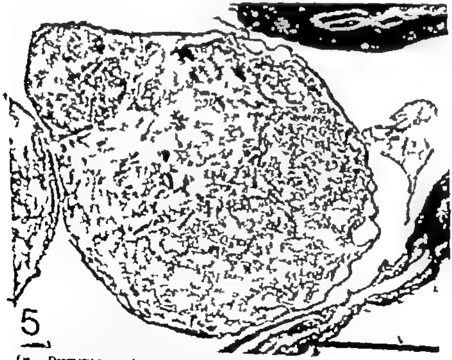

Fig. 5. Degenerating spiral ganglion cell from second turn in 90-day-old cat (no. 137). Note the eccentric lobulated nucleus and the single layer of Schwann cell cytoplasm with formation of spaces between this layer and the plasmalemma. Marker=5 μm.

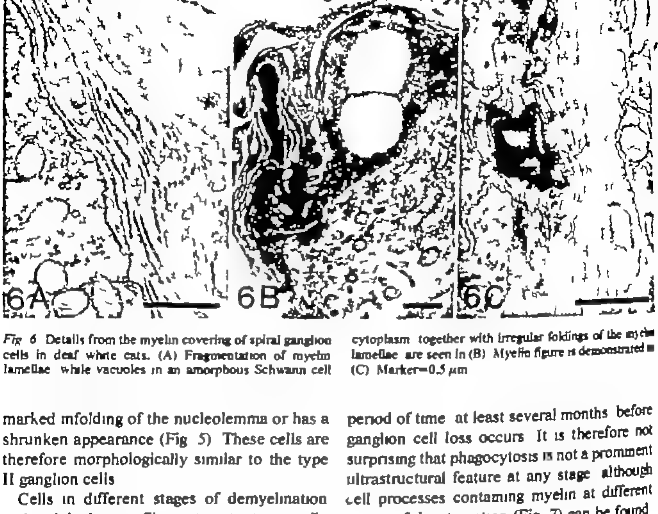

Fig 6 Details from the myelin covering of spiral ganglion cells in deaf white cats. (A) Fragmentation of myelin lamellae while vacuoles in an amorphous Schwann cell cytoplasm together with irregular foldings of the myelin lamellae are seen in (B) Myelin figure is demonstrated in (C) Marker=0.5 µm

marked infolding of the nucleolemma or has a shrunken appearance (Fig 5) These cells are therefore morphologically similar to the type II ganglion cells

Cells in different stages of demyelination and with high neurofilament content are easily found in each preparation from the spiral ganglion after the age of 90 days Similar cells are found extremely rarely in hearing cats while there appears to be an absolute increase in their numbers in deaf animals In older but not elderly deaf white cats Rosenthal s canal presents an empty appearance due to a pronounced reduction in the number of ganglion cells with a corresponding increase in the extracellular interstitial space These changes are however not found equally throughout the ganglion Even in 6 year-old deaf animals there is appreciably greater neuronal preservation at the extreme ends of Rosenthal s canal although unmyelinated filamentous neurons can be readily identified in these regions

The process of neuronal degeneration is initiated by destruction of the cochlear sensory cells and proceeds slowly over a considerable period of time at least several months before ganglion cell loss occurs It is therefore not surprising that phagocytosis is not a prominent ultrastructural feature at any stage although cell processes containing myelin at different stages of disintegration (Fig 7) can be found

DISCUSSION

Electron microscopic studies have revealed the existence of two and occasionally three different neuronal populations in the spiral ganglion of goldfish (Rosenbluth & Palay 1961) the rat (Rosenbluth 1962 Ross & Burkel 1973 Merck et al 1977) guinea pig (Kellerhals et al 1967 Thomsen 1967 Rossi et al 1976 Merck et al 1977) and the cat (Spoendlin 1971 Adamo & Daigneault 1973) In the majority of instances the neurons have been differentiated into a myelinated group with granular cytoplasm (type I) and a numerically smaller population of non myelinated ganglion cells with greater cytoplasmic content of neurofilaments (type II) Two different types of myelinated ganglion cells have also been described in the rat and cat (Rosenbluth

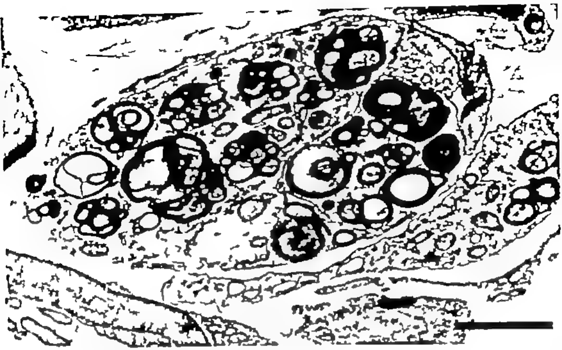

Fig. 7. Irregular cellular processes containing numerous vacuoles and myelin-bodies. Marker= μm

196., Adamo & Daigneault 1973) the differentiation again being based on variations in the content of both neurofilaments and rough endoplasmic reticulum.

There is considerable inter-species variation in the relative ease with which the unmyelinated type II ganglion cells can be located in the spiral ganglion of normal hearing animals (Kellerhals et al. 1967, Spoendlin 1971). It was only after electron microscopic study of a great many sections that these cells were first observed in the hearing cat (Spoendlin 1971). The retrograde degeneration (which occurs following intracranial transection) of all axons in the cochlear nerve produces loss of most spiral ganglion cells and inner hair cell afferent fibres, whilst the afferent fibres and synapses of the outer hair cells persist unchanged even after observation periods of up to 14 months (Spoendlin & Gacek 1963, Spoendlin 1974). The majority of these surviving spiral ganglion cells have the same morphological characteristics as the unmyelinated type II cells found so rarely in hearing animals; in the cat these type II ganglion cells have been estimated to account for only 5% of the total neuronal population of Rosenthal's canal and constitute the entire afferent innervation of the outer hair cells (Spoendlin 1971, 1974, 1978).

Neural degeneration and spiral ganglion cell loss also occur following a variety of both endogenously and exogenously induced damage to the cochlear receptor organ (Kerr & Schuknecht 1968). Most studies on acoustic hyperstimulation and ototoxicity have shown a greater vulnerability of the outer hair cells (Ward & Duvall 1971, Ades et al. 1974, Ylikoski 1974, Lim 1976) whilst retrograde degeneration of the afferent cochlear fibres occurs first when the inner hair cells or their afferent dendrites are affected and/or collapse of the supporting elements of Corti's organ has occurred (Schuknecht 1953, Spoendlin 1971, 1975, Ward & Duvall 1971, Ylikoski 1974, Lim 1976). Transmission electron microscopical studies of acoustically damaged ears have indicated a greater vulnerability of the afferent fibres and endings on the inner hair

cells whilst the corresponding structures persist even after severe damage and loss of the outer hair cells (Spoendlin 1971) These findings together with the observed persistence of the unmyelinated type II spiral ganglion cells in severely damaged cochleas (Spoendlin 1971 Lim 1976) would support the view that the type II neurons innervate only the outer hair cells However silver or zinc iodide–osmic acid staining of surface preparations which provides a more comprehensive picture of cochlear innervation patterns has revealed loss of tunnel-crossing fibres and afferent nerve endings and fibres in areas where the outer hair cells have been severely damaged by noise or ototoxic antibiotics (Engström et al 1966 Wright 1976) That retrograde degeneration of the outer hair cell afferents occurs is also indicated by the findings in the later stages of degeneration in the deaf white cat where the basilar membrane is totally denuded of all cellular and neural tissue (Mair 1973) Degeneration of the afferent fibres from the outer hair cells is therefore also accompanied by an increase in at least the relative numbers of unmyelinated ganglion cells

Several electron microscopic studies have been published on spiral ganglion cells following exposure to acoustic overstimulation (Awataguchi et al 1965 Kellerhals et al 1967 Rossi et al 1976) and overdosage with ototoxic antibiotics (Kellerhals et al 1967 Ylikoski 1974) These agents have resulted in dilatation of mitochondria and endoplasmic reticulum increased numbers of multivesicular and pigment bodies vacuolization of myelin sheaths and formation of myelin figures The relative extent to which the degeneration process affects the myelinated and unmyelinated ganglion cells has varied considerably A greater susceptibility of the latter has been reported (Awataguchi et al 1965) whilst other studies have indicated either a similar susceptibility of the two neuronal types (Kellerhals et al 1967) or better preservation of the unmyelinated cellular population (Ylikoski 1974 Rossi et al 1976)

In cochleas with pronounced neuronal degeneration secondary to destruction of Corti s organ the surviving ganglion cells show an increase in the relative numbers of unmyelinated type II neurons (Spoendlin 1971 1974 1975 Lim 1976) Both type I and type II ganglion cells are present in the spiral ganglion of older deaf waltzing guinea pigs although no estimation of the relative numbers was made in a recent study (Gulley et al 1978) Type II cells predominated in the depleted spiral ganglion of cats following neomycin overdosage and electrode implantation (Schindler & Björkroth 1979) In this context the study of Ylikoski (1974) is of particular interest the spiral ganglion from guinea pigs treated with overdosage of ototoxic antibiotics contained an abundance of unmyelinated ganglion cells. Indeed in the animal with longest post-treatment survival period the unmyelinated neurons accounted for 50% of the surviving ganglion cells (Ylikoski 1974) Since neuronal survival was estimated at ¼–⅜ the number of unmyelinated ganglion cells was probably of the order of 15–30% of the normal total ganglion cell population this being appreciably greater than the figures usually cited for the proportion of these cells in the spiral ganglion of this species (Kellerhals et al 1967 Thomsen 1967 Merck et al 1977) Ylikoski (1974) concluded that the unmyelinated ganglion cells could represent neurons which had lost their myelin sheaths in the degeneration process, while simultaneously developing an increased content of neurofilaments

An increase in the cytoplasmic content of neurofilaments is one of the well-recognized types of neuronal degeneration (Guillery 1970 Raisman & Matthews 1972) and has been reported inter alia in the neighbouring Scarpa s ganglion following post-ganglionic nerve section (Fuentes & Raymond 1972) and in the facial nucleus (Torvik 1977) efferent cochlear nerve endings (Smith & Rasmussen 1965) colliculus inferior (Jones & Rockel 1972) and red nucleus (Barron et al 1975) secondary to neuronal transection

Neurofilament accumulation has also been described following experimental spinal cord trauma (Balentine 1978) and in a wide variety of neuropathies (Koch et al 1977) The cytoplasm of the unmyelinated type II spiral ganglion cells contains numerous neurofilaments in contrast to the granular appearance of the type I myelinated perikarya (Thomsen 1967 Spoendlin 1971 1974 Adamo & Daigneault 1973). Perinuclear accumulation of rough endoplasmic reticulum has also been interpreted as a sign of degeneration in spiral ganglion cells (Kellerhals et al 1967 Ylikoski 1974) but is an ultrastructural feature of the unmyelinated neurons (Ross & Burkel 1973) Eccentrically sited nucleus infolding of the nuclear membrane and increase in the cytoplasmic content of neurofilaments autophagic vacuoles and multilamellar dense bodies also occur in the degenerating sensory root ganglia of Sprawling mice and have been interpreted as manifestations of a genetic defect acting directly on the sensory ganglion cells (Duchen & Scaravilli, 1977)

Following transection of the cochlear nerve in the internal auditory meatus a third group of spiral ganglion cells has been described these type III ganglion cells are morphologically similar to the type I with a rounded nucleus and few neurofilaments but the cell body is unmyelinated (Spoendlin 1974 1978) It should be noted here that some of the type II ganglion cells found in the normal cat have also a thin myelin sheath although this is uncommon (Spoendlin 1978) After short survival periods of 4 months following transection of the stato-acoustic nerve (Spoendlin & Gacek 1963) the type II and III ganglion cells have been found to constitute almost equal numbers of the surviving neurons in Rosenthal's canal (Spoendlin 1974). However when the postoperative survival period was extended to years the numbers of the type III cells became progressively reduced until almost all the surviving neurons were represented by a "normal population of type II cells (Spoendlin & Suter 1976 Spoendlin 1978) Following complete destruction of Corti's organ most spiral ganglion cells loose their myelin sheaths the nuclear membrane becomes folded and the cells are difficult to distinguish from type II neurons although being designated as type III (Spoendlin 1978 1979) The precise identity of and interrelationship between the three types of ganglion cells, and possible intermediate forms, would therefore appear somewhat uncertain at present.

We have been impressed both by the ease with which unmyelinated ganglion cells may be found in the spiral ganglion of the hereditarily deaf white cat and by the absolute numbers of these cells in degenerating ears and would suggest that these unmyelinated filamentous ganglion cells represent one of the commoner forms of neuronal degeneration in the spiral ganglion of the cat, the type I cells transforming into type II through an intermediate type III stage The dynamic changes in numerical relationships of the latter two neuronal types following nerve transection (Spoendlin 1978) would also support this interpretation We have confirmed earlier findings (Mair 1973 Elverland et al 1977) that ganglion cells loss is greatest in the upper basal and second coils and increases with advancing age but always with greater neuronal preservation at both extremities of Rosenthal's canal Ganglion cell loss is a late manifestation of the degeneration process, since the first statistical evidence of reduction in neuronal population was found after the age of 10 months (Mair 1973) However definite evidence of an increase in the numbers of unmyelinated ganglion cells has now been found at the 3-month stage in the present investigation Primary neural degeneration (Pujol et al 1977) has not been observed in our stock of hereditarily deaf white animals.

ACKNOWLEDGEMENTS

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cells whilst the corresponding structures persist even after severe damage and loss of the outer hair cells (Spoendlin 1971) These findings together with the observed persistence of the unmyelinated type II spiral ganglion cells in severely damaged cochleas (Spoendlin 1971 Lim 1976) would support the view that the type II neurons innervate only the outer hair cells However silver or zinc iodide–osmic acid staining of surface preparations which provides a more comprehensive picture of cochlear innervation patterns has revealed loss of tunnel-crossing fibres and afferent nerve endings and fibres in areas where the outer hair cells have been severely damaged by noise or ototoxic antibiotics (Engström et al 1966 Wright 1976) That retrograde degeneration of the outer hair cell afferents occurs is also indicated by the findings in the later stages of degeneration in the deaf white cat where the basilar membrane is totally denuded of all cellular and neural tissue (Mair 1973) Degeneration of the afferent fibres from the outer hair cells is therefore also accompanied by an increase in at least the relative numbers of unmyelinated ganglion cells

Several electron microscopic studies have been published on spiral ganglion cells following exposure to acoustic overstimulation (Awataguchi et al 1965 Kellerhals et al 1967 Rossi et al 1976) and overdosage with ototoxic antibiotics (Kellerhals et al 1967 Ylikoski 1974) These agents have resulted in dilatation of mitochondria and endoplasmic reticulum increased numbers of multivesicular and pigment bodies vacuolization of myelin sheaths and formation of myelin figures The relative extent to which the degeneration process affects the myelinated and unmyelinated ganglion cells has varied considerably A greater susceptibility of the latter has been reported (Awataguchi et al 1965) whilst other studies have indicated either a similar susceptibility of the two neuronal types (Kellerhals et al 1967) or better preservation of the unmyelinated cellular population (Ylikoski 1974 Rossi et al 1976)

In cochleas with pronounced neuronal degeneration secondary to destruction of Corti s organ the surviving ganglion cells show an increase in the relative numbers of unmyelinated type II neurons (Spoendlin 1971 1974 1975 Lim 1976) Both type I and type II ganglion cells are present in the spiral ganglion of older deaf waltzing guinea pigs although no estimation of the relative numbers was made in a recent study (Gulley et al 1978) Type II cells predominated in the depleted spiral ganglion of cats following neomycin overdosage and electrode implantation (Schindler & Björkroth 1979) In this context the study of Ylikoski (1974) is of particular interest the spiral ganglion from guinea pigs treated with overdosage of ototoxic antibiotics contained an abundance of unmyelinated ganglion cells Indeed in the animal with longest post-treatment survival period the unmyelinated neurons accounted for 50% of the surviving ganglion cells (Ylikoski 1974) Since neuronal survival was estimated at ¼–⅓ the number of unmyelinated ganglion cells was probably of the order of 15–30% of the normal total ganglion cell population thus being appreciably greater than the figures usually cited for the proportion of these cells in the spiral ganglion of this species (Kellerhals et al 1967 Thomsen 1967 Merck et al 1977) Ylikoski (1974) concluded that the unmyelinated ganglion cells could represent neurons which had lost their myelin sheaths in the degeneration process while simultaneously developing an increased content of neurofilaments

An increase in the cytoplasmic content of neurofilaments is one of the well-recognized types of neuronal degeneration (Guillery 1970 Raisman & Matthews 1973) and has been reported inter alia in the neighbouring Scarpa s ganglion following post-ganglionic nerve section (Fuentes & Raymond 1977) and in the facial nucleus (Torvik 1972) efferent cochlear nerve endings (Smith & Rasmussen 1965) colliculus inferior (Jones & Rockel 1972) and red nucleus (Barron et al 1975) secondary to neuronal transection

Neurofilament accumulation has also been described following experimental spinal cord trauma (Balentine 1978) and in a wide variety of neuropathies (Koch et al 1977) The cytoplasm of the unmyelinated type II spiral ganglion cells contains numerous neurofilaments in contrast to the granular appearance of the type I myelinated perikarya (Thomsen 1967 Spoendlin 1971 1974 Adamo & Daigneault 1973). Perinuclear accumulation of rough endoplasmic reticulum, has also been interpreted as a sign of degeneration in spiral ganglion cells (Kellerhals et al. 1967 Ylikoski 1974) but is an ultrastructural feature of the unmyelinated neurons (Ross & Burkel 1973) Eccentrically sited nucleus infolding of the nuclear membrane and increase in the cytoplasmic content of neurofilaments autophagic vacuoles and multilamellar dense bodies also occur in the degenerating sensory root ganglia of Sprawling mice and have been interpreted as manifestations of a genetic defect acting directly on the sensory ganglion cells (Duchen & Scaravilli 1977)

Following transection of the cochlear nerve in the internal auditory meatus a third group of spiral ganglion cells has been described these type III ganglion cells are morphologically similar to the type I with a rounded nucleus and few neurofilaments but the cell body is unmyelinated (Spoendlin 1974 1978) It should be noted here that some of the type II ganglion cells found in the normal cat have also a thin myelin sheath although this is uncommon (Spoendlin 1978) After short survival periods of 4 months following transection of the stato-acoustic nerve (Spoendlin & Gacek 1963) the type II and III ganglion cells have been found to constitute almost equal numbers of the surviving neurons in Rosenthal's canal (Spoendlin 1974) However when the postoperative survival period was extended to years the numbers of the type III cells became progressively reduced until almost all the surviving neurons were represented by a normal population of type II cells (Spoendlin & Suter 1976 Spoendlin 1978) Following complete destruction of Corti's organ most spiral ganglion cells loose their myelin sheaths the nuclear membrane becomes folded and the cells are difficult to distinguish from type II neurons although being designated as type III (Spoendlin 1978 1979) The precise identity of and interrelationship between the three types of ganglion cells and possible intermediate forms would therefore appear somewhat uncertain at present

We have been impressed both by the ease with which unmyelinated ganglion cells may be found in the spiral ganglion of the hereditarily deaf white cat and by the absolute numbers of these cells in degenerating ears and would suggest that these unmyelinated filamentous ganglion cells represent one of the commoner forms of neuronal degeneration in the spiral ganglion of the cat, the type I cells transforming into type II through an intermediate type III stage The dynamic changes in numerical relationships of the latter two neuronal types following nerve transection (Spoendlin 1978) would also support this interpretation We have confirmed earlier findings (Mair 1973 Elverland et al 1977) that ganglion cells loss is greatest in the upper basal and second coils and increases with advancing age but always with greater neuronal preservation at both extremities of Rosenthal's canal Ganglion cell loss is a late manifestation of the degeneration process, since the first statistical evidence of reduction in neuronal population was found after the age of 10 months (Mair 1973) However definite evidence of an increase in the numbers of unmyelinated ganglion cells has now been found at the 3-month stage in the present investigation Primary neural degeneration (Pujol et al 1977) has not been observed in our stock of hereditarily deaf white animals.

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ZUSAMMENFASSUNG

Das Spiralganglion von mit erblicher Taubheit belasteten weißen Katzen wurde mit dem Transmissionselektronen mikroskop untersucht und mit normal hörenden Tieren verschiedenen Alters verglichen Ganglienzellenverlust erscheint sekundär zur Zerstörung des Cortischen Organs aber erst nach Verlauf einiger Monate Vor dem Neuronenverlust verlieren die Typ-I-Ganglienzellen ihre Myelinhülle und gleichzeitig entwickelt sich erhöhter Anteil an Neurofilamenten Die Typ-I-Ganglienzellen transformieren sich zu Typ II über ein Intermediärstadium des Typ III Dieser Prozeß der neurofilamentären Degeneration erscheint langsam und die Phagocytose ist daher wenig ins Auge fallend

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I. W. S. Mair
Dept. of Otorhinolaryngology
Regionsykehuset
University of Tromsø
Norway

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SCANNING ELECTRON MICROSCOPIC OBSERVATIONS ON THE DISTENDED REISSNER S AND SACCULAR MEMBRANES IN THE GUINEA PIG

Naoko Shinozaki and Robert S Kimura

From the Department of Otolaryngology Massachusetts Eye and Ear Infirmary and Harva d Medical School Boston Massachusetts USA

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Abstract The surface ultrastructures of membranous labyrinths in normal and hydropic guinea pig inner ears were studied In normal specimens differences between Reissner's and saccular membranes were observed The endolymphatic epithelial cells of the saccule were smaller in size and their microvilli varied more in size and distribution Mesothelial cells of the saccule were often spherical in shape and bulged toward the vestibule In hydropic guinea pig inner ears distension of Reissner s and saccular membranes occurred by irregular enlargement of epithelial cells. Microvilli were reduced in size and number On the severely distended Reissne s membrane outpouchings infoldings and holes were observed Transmission electron microscopic examination revealed no significant difference between the tight junctions of epithelial cells in normal or hydropic Reissner's membranes Mesothelial cells of Reissner s membrane were more severely degenerated than those of the saccule Cochlear sensory cells were often degenerated in the apical turn where mesothelial cells were lacking but saccular sensory cells showed very little change in thei surface structures

Experimental hydrops in animals has been studied in relation to the possible etiology of Meniere s disease (Naito 1959 Kimura & Schuknecht 1965 Kimura 1967 Schuknecht et al 1968 Konishi & Kelsey 1973 1976 Silverstein & Takeda, 1977) Reissner s and saccular membranes are the most distended membranes in the inner ears of Meniere s disease specimens and in the inner ears of animals with experimentally induced hydrops Both membranes consist of two cell groups the epithelial cells of ectodermal origin facing the endolymph and the mesothelial cells of mesenchymal origin facing the perilymph

The distended Reissner s membrane was studied in the guinea pig (Kimura 1967) and human (Kimura 1976 Kimura et al 1976) by transmission electron microscopy The reports indicated a frequent lack of mesothelial cells approximating the perilymph over an extensive area in the apical turns of the cochlea Atrophy of endolymphatic epithelial cells in some areas was also shown In a surface preparation study of distended Reissner s membrane in man Johnsson (1971) reported the possibility of cell multiplication as well as epithelial cell lesions

The purpose of the present investigation was to study the distended membranes in the experimental hydrops inner ear by both scanning (SEM) and transmission (TEM) electron microscopy

MATERIALS AND METHODS

A total of 28 guinea pigs were used for the normal and hydrops studies Fifteen guinea pigs were used for endolymphatic sac and duct obliteration according to the procedure reported by Kimura and Schuknecht (1965) Only the right ear of each animal was operated the left ear served as an unoperated control The postoperative survival times of the specimens ranged from 1 day to 8 3 months

Under anesthesia the animals were decapitated and the temporal bones were removed The membranous labyrinth was immediately fixed by perfusion through th^ ^^^^^ ^^^ oval

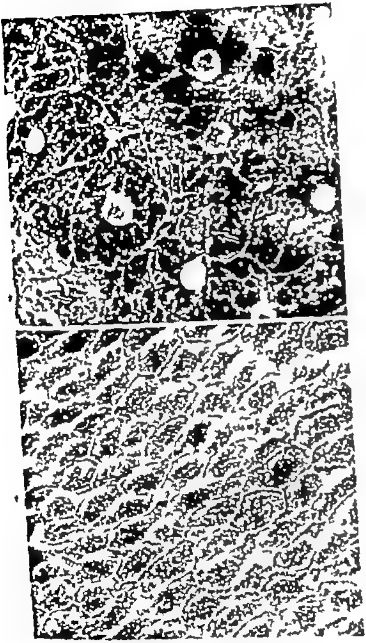

Fig. 1. Normal Reissner's membrane (control side). (A) Mesothelial cells show small holes (arrow) in the cytoplasm and gap (double arrow) between adjacent cells. ×1008. (B) Endolymphatic epithelial cells show clearly defined cell borders and numerous microvilli. Compare cell size with mesothelial cell size in (A). ×1008.

windows with 5% phosphate-buffered glutaraldehyde, postfixed with 1% phosphate-buffered osmium tetroxide and dehydrated up to 70% ethanol for dissection. After dissection, specimens were prepared by the OTOTO (osmium–thiocarbohydrazide repeated) method for SEM examination (Malick & Wilson 1975, Hunter-Duvar 1978). Specimens were rinsed in distilled water and immersed in a saturated filtered solution of thiocarbohydrazide (TCH) for 30 minutes. This step was followed by a 10-min rinse in distilled water and a one-hour immersion in 1% aqueous osmium tetroxide. The TCH and osmium

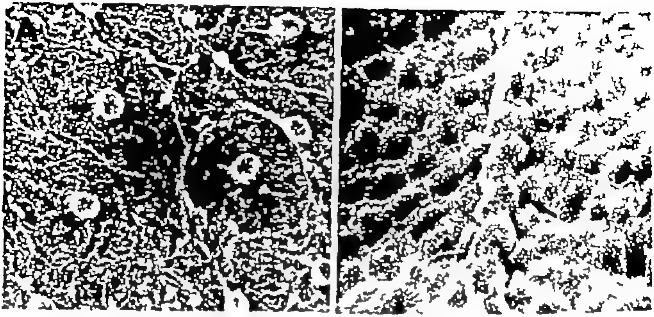

Fig 2 Distended Reissner's membrane in the apical turn 4.3 months postoperatively (A) On the perilymphatic surface a straight groove (*arrow*) is shown in the direction of the spiral ligament to the limbus spiralis ×750 (B) The opposite side of the groove as observed from the scala media side Epithelial cell ridge (*arrow*) coincides with the groove ×750

steps were repeated Specimens were then dehydrated through 100% ethanol dried in a critical point drier and mounted on stubs with adhesive aluminum tape The specimens were studied with a JEOL JSM 35 SEM For observation of both perilymphatic and endolymphatic surfaces of Reissner s and saccular membranes specimens were dissected again after the first observation with SEM

For TEM the specimens were dissected out after observation by SEM immersed in 100% ethanol followed by propylene oxide and embedded in Epon The specimens were sectioned on an LKB Ultrotome and examined with a Siemens Elmiskop 1 without staining

To observe the junctions of epithelial cells of Reissner s membrane the membranous labyrinth of one animal 8 3 months postoperative was fixed by perfusion through the round and oval windows with 1% phosphate-buffered osmium tetroxide immersed in fixative for 2 hours and rinsed with phosphate buffer and distilled water for 30 min respectively Specimens were immersed *en bloc* in 1% uranyl acetate in distilled water for 2 hours (Farquhar & Palade 1963) The tissue was dehydrated in graded ethanols exchanged with propylene oxide and embedded in Epon

FINDINGS

Reissner s membrane

The mesothelial cells on the perilymphatic side of normal as well as control Reissner's membranes varied in size (about 22×40 μm) and shape (Fig 1 A) The nucleus was round measured 6 5×7 4 μm and bulged into the perilymphatic space Small round holes in the cytoplasm 0 5 to 9 μm in diameter were observed in the mesothelial cells Gaps (about 4 μm) were sometimes observed between adjacent cells Some short microvilli (0.5 μm tall) were seen on the free surface but less than on the epithelial cells

The epithelial cells on the endolymphatic side of Reissner s membrane were slightly elongated in the radial direction and were pentagonal or more often hexagonal in shape (Fig 1B) These cells (8.3×22 μm) were smaller than the mesothelial cells Two or three rows of epithelial cells located at the limbus were small in comparison to other areas. There was no significant difference in shape

Fig. 3. (A) Perilymphatic surface of the distended Reissner's membrane third turn. One month postoperative. Mesothelial cells are stretched. Gaps between the cell junctions have become wider (arrow) and the basement membrane (BM) is exposed. Nuclear part of the epithelial cells can be seen as dark areas. ×960. (B) Perilymphatic surface of the distended Reissner's membrane apical turn, 4 months postoperatively. Mesothelial cells are degenerated and basement membrane is exposed. Many fibrils extend over the membrane mostly in radial direction. ×1680. (C) TEM of the same specimen as in (B). Note the absence of mesothelial cells on the perilymphatic side. ×1680.

size or arrangement throughout the cochlea. Many microvilli evenly covered the entire cell surface.

Reissner's membrane was distended from one day postoperative. In out of 15 specimens with survival times of 4, 3 and 6 months, some straight grooves (each 700 μm long) were observed under the mesothelial cells (Fig. A). These grooves extended in the direction of the spiral ligament to the limbus spiralis. Thin cytoplasmic processes of mesothelial cells crossed over the grooves. When the same membrane was examined from the endolymphatic side, ridges of the epithelial cells coincided with the grooves (Fig. 2 B).

In hydropic specimens, gaps between adjacent mesothelial cells gradually became larger as the postoperative time increased (Fig. 3 A). In severe hydrops (survival times of more than 3 months) mesothelial cells were extensively degenerated and a wide area of the basement

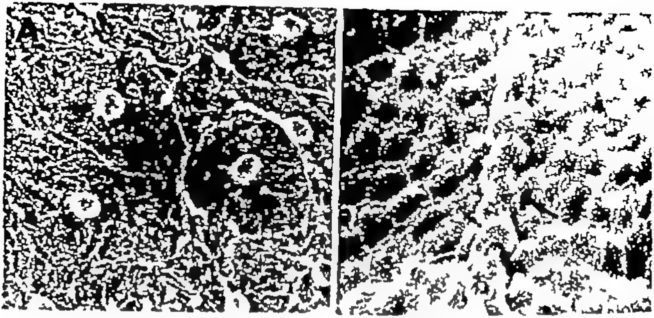

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Fig. 3. (A) Perilymphatic surface of the distended Reissner's membrane, third turn. One month postoperative. Mesothelial cells are stretched. Gaps between the cell junctions have become wider (arrow) and the basement membrane (BM) is exposed. Nuclear part of the epithelial cells can be seen as dark areas. ×960. (B) Perilymphatic surface of the distended Reissner's membrane, apical turn, 4 months postoperatively. Mesothelial cells are degenerated and basement membrane is exposed. Many fibrils extend over the membrane mostly in radial direction. ×1690. (C) TEM of the same specimen as in (B). Note the absence of mesothelial cells on the perilymphatic side. ×1690.

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Reissner's membrane was distended from one day postoperative. In 2 out of 15 specimens with survival times of 4, 3 and 6 months some straight grooves (each 700 µm long) were observed under the mesothelial cells (Fig. 3A).

These grooves extended in the direction of the spiral ligament to the limbus spiralis. Thin cytoplasmic processes of mesothelial cells crossed over the grooves. When the same membrane was examined from the endolymphatic side, ridges of the epithelial cells coincided with the grooves (Fig. 2B).

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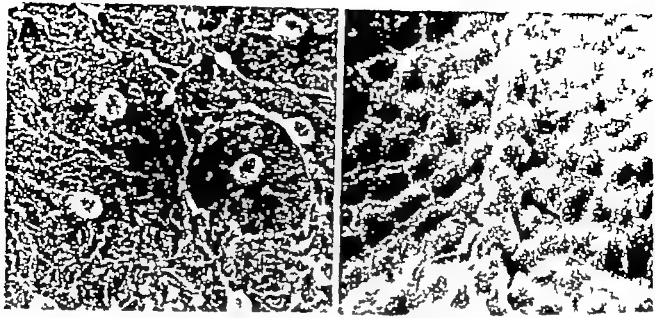

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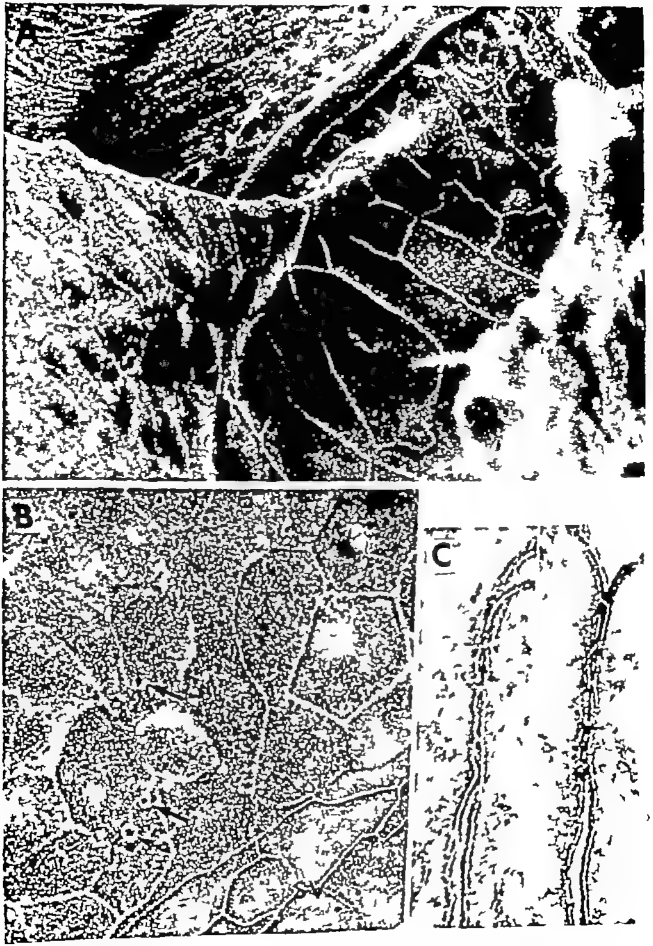
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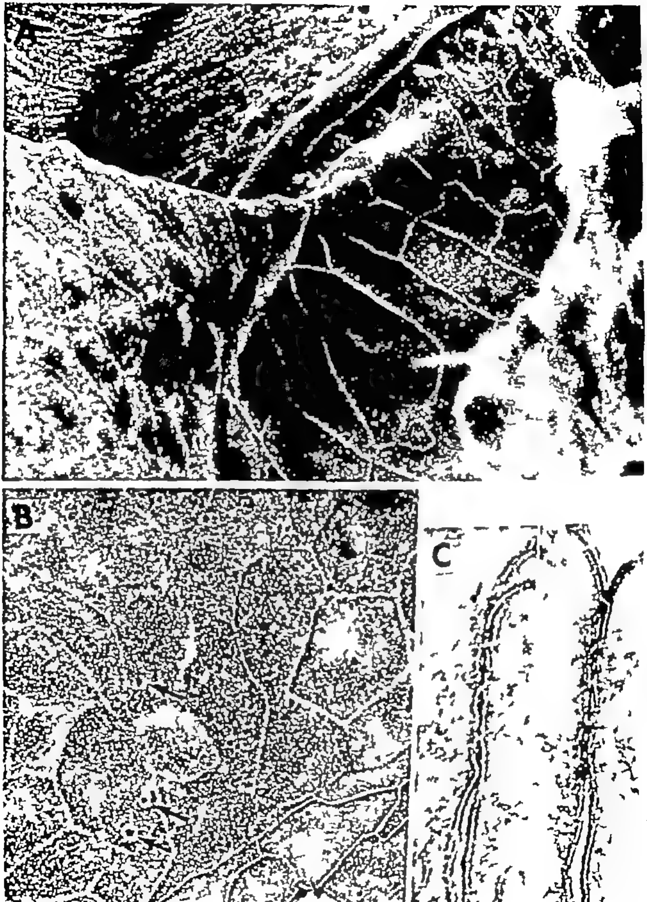
Fig. 5. (A) Outpouchings of Reissner's membrane near the limbus spiralis (arrows) in the basal turn 5 months postoperatively. Area marked by the square is shown in (B). ×220. (B) Higher magnification of the outpouching. The epithelial cells inside the pouch are more distended than those outside. ×1 900.

Fig. A. Distended Reissner's membrane near the limbus. Apical turn, 3.5 months postoperatively. Note moderately and severely distended epithelial cells. TM, tectorial membrane. ×930. (B) Distended Reissner's membrane near the stria vascularis. ×11. Note many pits (arrows). ×1100. (C) Tight junctions of the epithelial cells of Reissner's membranes show no significant difference. ×20000. *Left:* control side. *Right:* hydropic side. months postoperatively.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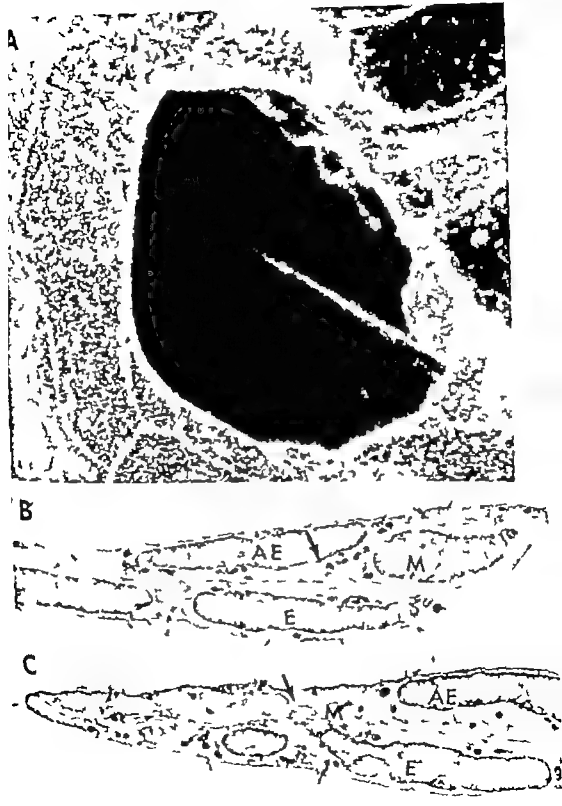
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membrane was exposed to the perilymphatic space (Fig 3B C) Degeneration was more severe in the apical turn and particularly more on the spiral ligament side than on the limbus side Many fibrils (50 to 100 Å) covered the basement membrane and extended radially in the direction of the limbus to the spiral ligament

The luminal surfaces of epithelial cells were enlarged in every turn of the cochlea from one day postoperative The magnitude of enlargement varied considerably from one part of the cochlea to another or even in the same region (Fig 4A) The cell size was enlarged almost two to four fold In the apical region where mesothelial cells were degenerated the enlargement of epithelial cells was not markedly different from that of other areas Small pits (0 5 to 1 0 μm) on the enlarged cells increased in number and microvilli decreased in density in comparison to control specimens (Fig 4B) When observed by TEM sealing strands in the tight junctions between epithelial cells of Reissner s membrane numbered 5 to 8 in both control and hydropic inner ears 8.3 months postoperative (Fig 4C) Pinocytotic vesicles in the epithelial cells increased slightly in number

One specimen (5 months postoperative) showed small outpouchings toward the perilymphatic side near the limbus in the basal turn (Fig 5A) The epithelial cells inside the pouches were more distended than those outside (Fig 5B) When they were examined from the perilymphatic side the mesothelial cells of this area had an abnormally high number of short microvilli Another specimen (4 months postoperative) showed small round holes (25× 35 μm) in the apical turn (Fig 6A) TEM study of the areas peripheral to one hole showed epithelial cells with basement membrane on the organ of Corti side whereas on the opposite side of the membrane the cells were atypical and were directly abutted by mesothelial cells without a basement membrane and in some areas the mesothelial cells were completely missing (Fig 6B C)

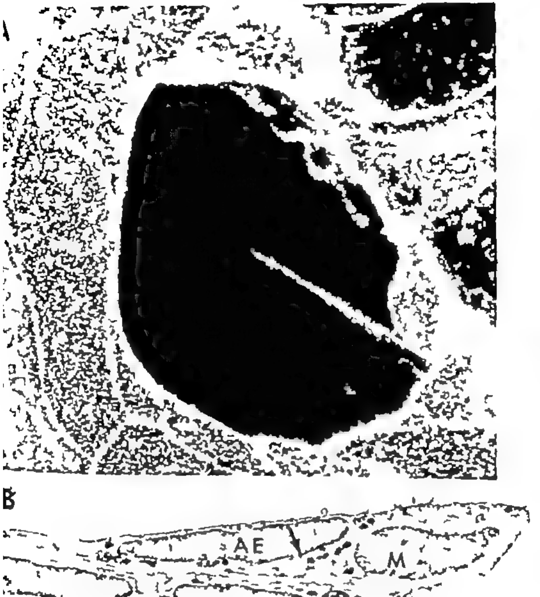
Organ of Corti

Cochleae in which Reissner s membranes were severely distended and the mesothelial cells were lacking over wide areas showed lesions of the outer and inner hair cells. In the apical turns (Fig 7) almost all of the outer hair cells were abnormal as well as severely degenerated Stereocilia of the hair cells were decreased in number or fused together The cuticular plates of the outer hair cells had disappeared and were replaced by phalangeal processes of the supporting cells In the third turns the inner hair cells appeared to be normal and outer hair cells were degenerated The outer hair cells of the outermost row were most frequently affected In the second and basal turns both inner and outer hair cells were normal Although the above pattern of degeneration was most common the extent of pathology varied among the specimens of different or equal survival times

Saccular membrane

Although the mesothelial cells of the normal saccular membrane resembled those of Reissner s membrane there were differences. There were balloon shaped mesothelial cells in addition to the usual flat type The cytoplasms of these cells were thin (0 15 μm) and appeared to surround large oval spaces (1? to 20 μm in diameter) (Fig 8A B) These cells in profile appeared to contain one or several large vacuoles but these spaces were open toward the basement membrane The spherically shaped cells were distributed over the en-

Fig 6 (A) SEM of the hole in the distended Reissner s membrane in the apical turn The tectorial membrane (TM) can be seen through the hole ×2900. (B C) TEM of the same area as shown in (A) (B) Shows the border on the left side of the hole in (A) (C) Shows the edge located close to the limbus on the right side of (A) The cells on the surface facing the tectorial membrane show epithelial cells (E) with a basement membrane which is demonstrable at higher magnifications The cells (AE) on the other surface of the membrane are atypical and are directly abutted by mesothelial cells (M) and are not underlined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Although the mesothelial cells of the normal saccular membrane resembled those of Reissner s membrane there were differences. There were balloon shaped mesothelial cells in addition to the usual flat type The cytoplasms of these cells were thin (0 15 μm) and appeared to surround large oval spaces (12 to 20 μm in diameter) (Fig 8A B) These cells in profile appeared to contain one or several large vacuoles but these spaces were open toward the basement membrane The spherically shaped cells were distributed over the en-

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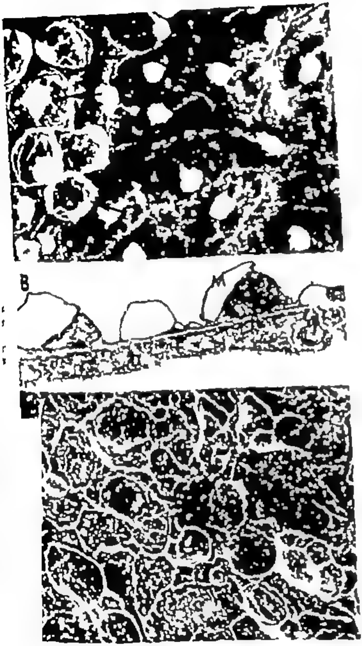

Fig. 8. Normal and distended mesothelial cells of saccular membranes. (A) The balloon-shaped (arrow) and flat mesothelial cells on the perilymphatic surface. ×790. (B) Cytoplasms of mesothelial cells (M) from normal specimens are thin and large vacuolar spaces are shown. However these vacuolar spaces are often open toward the basement membrane. One such example is shown on the right (arrow). E, endolymphatic epithelial cell. ×1580. (C) Severely distended part of the saccular membrane in hydropic inner ear. Basement membrane (BM) is exposed and fibrils are shown. ×948

After weeks postoperative survival time no normal epithelial cells were seen. The surface structures of the most distended epithelial cells were similar in early and late stages of hydrops. The most distended epithelial cells were 17.3×33.5 μm which was almost three times larger than in control specimens (Fig. 9C). The nuclear part of the cell bulged slightly and microvilli were unevenly distributed or decreased in number. Small pits (0.5 to 1 μm) were seen on the surface. Pinocytotic vesicles increased in number. Sensory cells of the saccule showed normal surface structure in the severely hydropic inner ear.

Fig 7 Organ of Corti of apical turn in severe hydrops, 3.3 months post operatively Stereocilia of inner (*IHC*) and outer (*OHC*) hair cells are fused and many outer hair cells are degenerated

tire saccular membrane except at the border of the anterior part of the saccular membrane Their nuclei (5 5×7 4 μm) were randomly located within the cell In the area adjacent to the spherical mesothelial cells the epithelial cells often showed plasma membrane infoldings at their basal surfaces No holes were seen in the cytoplasm and gaps were rarely shown between spherical or flat mesothelial cells

The epithelial cells of the endolymphatic surface were pentagonal or hexagonal in shape (Fig. 9 A) Cell size (average 9 3×10 9 μm) and surface structure varied not only from specimen to specimen but also in different parts of the same specimen The membrane showed clusters of small epithelial cells surrounded by larger cells Epithelial cells had many microvilli which were regular in size shape and arrangement but the variations were much greater than those of Reissner s membrane

Figure 9B shows abnormal otoconia attached to epithelial cells of a normal specimen The otoconia have become translucent and have lost their two pointed ends showing a round and rough surface Thin cytoplasmic processes of epithelial cells partly surrounded some otoconia which suggests phagocytic activity These epithelial cells differed from utricular dark cells which are known to phagocytose otoconia Dark cells were not observed in the saccule

Distension of the saccular membrane was observed one day postoperatively In both early and late stages of hydrops the location of distension or the location of maximum distension was not constant in all specimens In the distended membrane most of the vacuolar bulges of the mesothelial cells had collapsed or had become less obvious as hydrops became more severe Gaps developed between the mesothelial cells and the basement membrane and fine fibrils were exposed to perilymph (Fig 8C) However these gaps were small and were less frequently seen than in Reissner s membrane

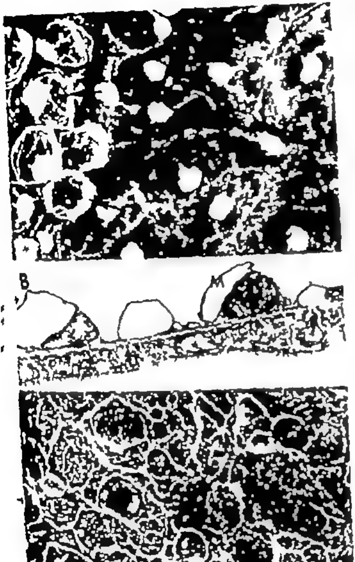

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Fig 9 (A) Normal saccular epithelial cell show abundant macrovilli. ×1 659 (B) Normal specimen showing otoconia attached to the saccular epithelial cells Otoconia are transparent and appear to be in the process of being phagocytosed ×3 160 (C) Severely distended saccular epithelial cells which are attached to the footplate of the stapes Compare cell size with (A) ×1 659

Stria vascularis and dark cells

Marginal cells of the stria vascularis in the control animal were pentagonally or hexagonally shaped and averaged 8 4×11 8 μm in size (Fig 10 A) No significant structural differences were observed along the cochlear duct There were many microvilli though fewer than on the epithelial cells of Reissner s and saccular membranes Microvilli uniformly covered the entire cell surface and some small pits were observed The cell borders were more elevated The dark cells of the utricle were about the same size as the marginal cells and their surface structures were similar to that of the marginal cells There were many small pits and normal-appearing and fragmented otoconia attached to the dark cells

In the hydropic ear which showed degenera

Fig. 10. (A) Normal stria vascularis (control side). Marginal cells are regularly shaped and covered with many microvilli. (B) Stria vascularis of the apical turn in severely hydropic inner ear. Marginal cells are larger than normal and the surface is flat. Microvilli are decreased in density. The same magnification as (A). ×1900.

tion of mesothelial cells, the surface structure of the marginal cells in the apical and third turns was flat (Fig. 10B). Microvilli were decreased in number and the height of cell borders was reduced. The marginal cells showed an enlarged surface (10.8×15.4 μm average) but the increase in size varied from cell to cell. Dark cells of the utricle were enlarged in a similar manner. The number of microvilli was decreased. However, these changes were less in the dark cells than in the marginal cells.

DISCUSSION

Some morphological differences between epithelial cells of Reissner's membrane and saccular membrane were observed in normal and control specimens. The mesothelial cells of the saccule rarely showed gaps between adjacent cells, but those of Reissner's membrane more frequently did. The long axis of epithelial cells in Reissner's membrane was aligned in the radial direction and it was about twice as great as that of the saccular epithelial cells. The shape and size of cells and distribution and length of microvilli on Reissner's epithelial cells remained the same along the cochlear duct, the microvilli varied more on the saccular epithelial cells. The average size of saccular epithelial cells was smaller. Another difference was the spherical bulge of many saccular mesothelial cells toward the vestibule. Their cytoplasm was almost identical with that of the flat mesothelial cells. The opening of the vacuolar space always faced the endolymphatic epithelial cells which sometimes showed basal infoldings; also these spherical cells were collapsed in the hydropic saccule. These spherical cells may represent a functional alteration of mesothelial cells to engage in phagocytic activity or they may play a part in regulating fluid transport activity between the endolymphatic and perilymphatic compartments. Similar vacuolar cells were described in Schlemm's canal of the eye (Inomata et al. 197?).

Reissner's and saccular membranes were distended by enlargement of cells. The most

distended epithelial cells were two to four times larger than normal in both Reissner s and saccular membranes However the epithelial cells of the saccular membrane were often more distended Johnsson (1971) observed epithelial cell lesions and many small epithelial cells in the distended Reissner s membrane of a specimen from a patient with hydrops He suggested the possibility of cell multiplication as well as epithelial cell degeneration Using surface preparation and autoradiographic techniques Watanuki (1968) and Watanuki et al (1968) reported that the epithelial cells of Reissner s membrane were smaller and more active on the limbus side than on the spiral ligament side In this study small epithelial cells of Reissner s membrane at corresponding locations in hydropic ears did not increase in number instead they were enlarged There was no morphological evidence to suggest cell multiplication nonetheless cell division could have occurred

In hydropic inner ears there were wide gaps between mesothelial cells of both Reissner s and saccular membranes The mesothelial cells of Reissner s membrane normally show small gaps (Duvall & Rhodes 1967) or pores (Hunter Duvar 1978) Although saccular epithelial cells were equally or more severely distended than Reissner s epithelial cells there was less degeneration of saccular mesothelial cells and atrophic changes in sensory cells were not obvious On the other hand cochlear hair cells were often atrophic in the area of degenerated mesothelial cells The reason is not clear though it could be related to morphological differences and/or it may be related to differences in chemical composition developed at these locations

In the hydropic inner ears of guinea pigs at 5 to 10 weeks survival times a slight increase in the Na^+ content of endolymph was reported by Konishi & Kelsey (1973) Silverstein & Takeda (1977) in a similar experiment showed significant increase in Na and a decrease in K concentrations in endolymph 8 to 16 weeks postoperatively Whether these chemical changes caused degeneration of mesothelial cells and sensory cells or whether they were due to changes in the distended membranes is obscure at present On the other hand Morgenstern & Miyamoto (1979) found no increase in K^+ of perilymphatic fluid in 14-day and 3-month postoperative specimens

Rupture and repair of the membranous wall were frequently shown in the inner ears of Meniere s disease patients (Altmann & Kornfeld 1965 Schuknecht 1975) In the present study a possible rupture was shown in the apical turn (Fig 6A B C) SEM of the area peripheral to the hole viewed from the apical direction after removal of the bony wall showed cell surfaces which resembled epithelial cells However under TEM these cells were atypical they were abutted by mesothelial cells and lacked a basement membrane The presence of these atypical cells may suggest cellular alteration as a result of fistulization The straight grooves were oriented in the radial direction as were the fibrils on the basilar membrane Reissner s membrane may be weaker in this direction and may tear The healed fistula could be shown as a groove in the perilymphatic side and as an infolding in the endolymphatic side The other possibility is that an irregular distension of the membrane caused this infolding

It is generally thought that the main function of tight junctions is to enable a group of cells to maintain an internal environment that differs from the external one (Staehelin & Hull 1978) Evan et al (1976) in their study of nephrons which were subjected to expansion by blood or fluid volume reported no detectable alteration in the fine structure of the tight junction The present study revealed no significant changes in the tight junctions of the hydropic inner ear after a long survival time therefore it does not suggest that the membrane became leaky at this cell junction A change might have occurred at an earlier stage of hydrops and later recovered Microvilli were reduced in number however pinocytotic activity appeared somewhat increased

From these observations it could not be deter mined whether there was an increase or de crease in fluid transport through the cytoplasm of epithelial cells.

Dark cells of the utricle are reported to participate in the removal of dislodged otoconia (Lim 1973 Harada & Sugimoto 1977) No dark cells were found in the saccule in the present study as in the earlier report by Kimura (1969) This study showed that some otoconia, presumably dislodged from the otolithic membrane of the saccule were attached to the saccular epithelial cells and appeared to be phagocytosed by these cells in both normal and hydropic ears The saccular membrane in this study did not reveal fragmented or globular structures of otoliths which are typical in the dark cells The means of disposing of otoconia might differ in dark cells versus the saccular epithelial cells but a more detailed study is needed. Gussen (1978) reported displacement of otoconia from the degenerated macula sacculi to the cochlear duct through the ductus reuniens She suggested that such displacement produced secondary degeneration of cochlear structures In the present study no otoconia were found in the ductus reuniens or in the basal end of the cochlear duct It is not clear if saccular otoliths are transported to the endolymphatic sac Guild (1927) was uncertain of this aspect in the guinea pig, though he indicated the presence of numerous otolithic crystals and debris in the endolymphatic sac in the lower species

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ZUSAMMENFASSUNG

Die Ultrastruktur des membranösen Labyrinthes von normalen und hydropischen Innenohren des Meerschweinchens wurde untersucht. In normalen Präparaten wurden Unterschiede zwischen der Reißnerschen Membran und der Membran des Sacculus beobachtet. Die endolymphatischen Epithelzellen des Sacculus waren kleiner und ihre Mikrovilli variierten mehr in Größe und Verteilung. Mesothelzellen des Sacculus waren oft rund und in das Vestibulum vorgewölbt. I hydropischen Innenohren on Meerschweinchen erschien die Dehnung der Reißnerschen Membran und der Membran des Sacculus als unregelmäßige Vergrößerung von Epithelzellen. Mikrovilli waren in Zahl und Größe reduziert. An der stark gedehnten Reißnerschen Membran wurden Ausstülpungen, Einfaltungen und Löcher beobachtet. Die Untersuchung unter dem Transmissions-Elektronenmikroskop zeigte keine signifikanten Unterschiede der tight junctions von Epithelzellen in normalen oder hydropischen Reißnerschen Membranen. Die Mesothelzellen der Reißnerschen Membran waren mehr degeneriert als die des Sacculus Sinneszellen der Cochlea waren in der Spitzenwindung häufig degeneriert, w Mesothelzellen fehlten. Sinneszellen im Sacculus zeigten sehr geringe V ränderungen ihrer Oberflächenstruktur

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Robert S Kimura Ph.D
Department of Otolaryngology
Massachusetts Eye and Ear Infirmary
243 Charles Street
Boston Massachusetts 02114
USA

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STRUCTURE OF THE HAIR ROOTLETS ON COCHLEAR SENSORY CELLS BY TANNIC ACID FIXATION

M. Itoh and T. Nakashima

From the Department of Otorhinolaryngology, Faculty of Medicine, Kyushu University, Fukuoka, Japan

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Abstract. Rootlets of the sensory hairs of the basal turn of the guinea pig cochlea were investigated using the tannic acid fixation method under transmission electron microscopy (TEM). The fine structure of the rootlet was enhanced by adding tannic acid to the prefixative for the specimen. By using the tannic acid fixation method it is possible to observe the finer filamentous structure of the rootlet in greater detail than is revealed by using the ordinary glutaraldehyde–osmium fixation method. TEM observation by the tannic acid method reveals that the rootlet consists of 90 Å diameter protofilaments and an electron-dense background substance. These protofilaments are arranged in an extremely regular pattern. They are considered to be modified microfilaments.

The fine structure of the rootlets in cochlear sensory cells and lateral line organs has been described by several investigators (Engstrom et al. 1962, Flock 1965, Engstrom & Engström 1978). They described the structure as "dark delicate filaments", "dense fibrils" or "microfibrils". The inside structure of the hair and the rootlet, however, has not been fully explored. Hairs have been believed to act as levers, transmitting mechanical energy from the tectorial membrane to the cuticular plate (Engstrom et al. 196_). The physical movements of the sensory hairs are the first step in the cellular excitation process, and it is therefore of great importance to obtain detailed morphological and biochemical information on the hair and rootlet.

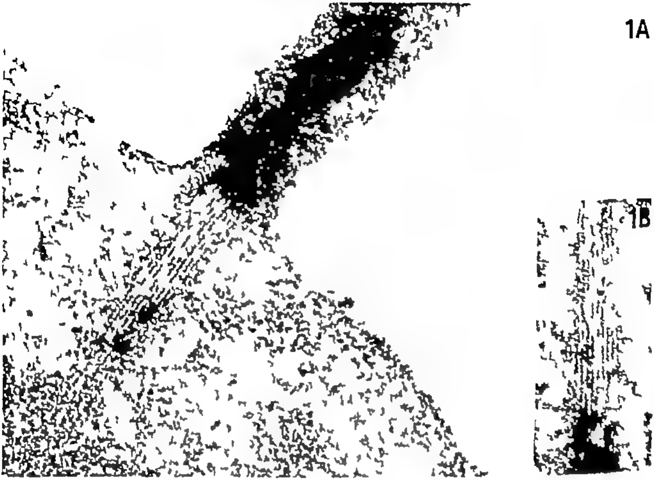
The present study is intended to provide a more detailed description of the structure using the tannic acid fixation method. This method was developed by Mizuhira & Futaesaku (1977), who studied microtubules in a cross section of the rat sperm tail and succeeded in identifying and counting the protofilaments.

METHODS

In this study, guinea pigs were anesthetized by subperitoneal injection of sodium pentobarbital and decapitated. The auditory bulla was immediately removed and the cochlea was fixed in a 1/14 M veronal acetate buffered glutaraldehyde–tannic acid solution.

A buffered 2.5% glutaraldehyde solution was used to dissolve the tannic acid, forming a 2.0% tannic acid solution. The final pH of the fixative was adjusted at 6.0. Sodium sulfoxide was added to the fixative, reaching a concentration of 1.0% to adjust the osmotic pressure. The cochlea was perfused by the fixative through openings in both the oval and the round windows. Small holes were then drilled in the scala media of the apex and basal portion of the specimen to allow free passage of the fixatives.

After 2 hours of fixation in glutaraldehyde–tannic acid solution at 4°C, the specimen was rinsed in a buffered 6% saccharose solution and postfixed in 1% osmium tetroxide with a 1/14 M veronal acetate buffer for 2 hours at 4°C. Dimethyl sulfoxide (DMSO) was added to the fixatives and rinse solution at a 1% concentration to facilitate the penetration of the fixatives into the cell. The specimen was dehydrated through a series of alcohol concentrations and embedded in Epon 812. Ultrathin



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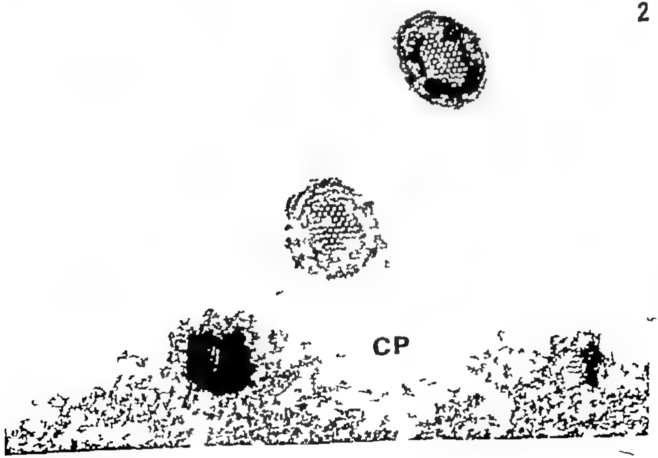


sections were doubly stained with uranyl acetate and lead citrate and observed with a JEOL JEM 100S electron microscope

RESULTS

The fine structures of the hair and its rootlet are shown in longitudinal section (Fig. 1 A B) The rootlet appears negatively stained' and contrast of the white lines is enhanced moving up from the cuticular plate into the basal portion of hair The author calls these white lines protofilaments. Each protofilament is arranged at equal distances of 75 Å The protofilaments are observed as white dots close to the cuticular surface in a cross-section of the specimen, as shown in Fig. 2 These dots correspond to the white lines of the longitudinal specimen About fifty white dots can be seen in the centre of each rootlet and hair They are arranged in a very regular manner Each dot is about 50 Å in diameter and the basic pattern of their arrangement forms a hexagon

Cross-sections taken from the cuticular plate reveal various sized rootlets as shown in Fig. 3 Higher magnification reveals two basic patterns Fig 4 A shows a solid cluster of protofilaments and Fig 4 B shows a cluster of protofilaments around a tubular centre Both types of cluster appear in an electron-dense substance surrounded by a lighter zone These protofilaments appear to be chained to one another by finer filaments There is a lighter zone which surrounds each rootlet and darker stripes are clearly observed radiating from each rootlet A cross-section of the protofilaments together with a schematical line defining their hexagonal pattern is shown in Fig 5 under much higher magnification A schematic drawing of the hexagonal pattern is shown in Fig. 6

The protofilaments in the basal portion of the hair extend down into the cuticular plate as shown in the schematic drawing in Fig. 7 The largest number of protofilaments are found in cross-sections taken from the cuticular surface Their numbers decrease gradually in sections moving up into the basal portion of hair and down into the cuticular plate

Fig 1 Hair on outer hair cell of the guinea pig Contrast of the [illegible] lines is enhanced in rootlet Same-sized protofilaments lying parallel to one another project down from the basal portion of hair int cuticular plate (A) Basal portion of hair 160 000 (B) Hair rootlet deep in cuticular plate 187 000

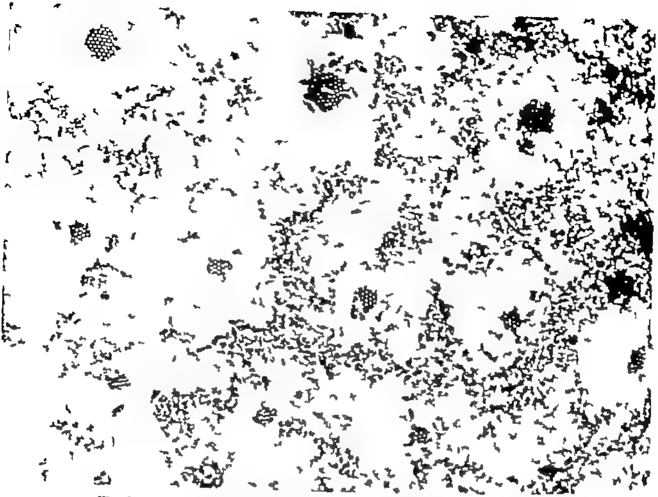
Fig 2 Cross-sectioned hairs from outer hair cell close to the cuticular plate (*CP*). About fifty protofilaments are arranged at equal distance to one another in the centre of each hair as white dots 300 000

DISCUSSION

Tannic acid precipitates with soluble proteins polypeptides alkaloids and metallic cations to a high degree in vitro By using glutaraldehyde–tannic acid fixation the subunit structures of microtubules were readily observed The number of protofilaments can be counted in the structure without the need of image reinforcement (Mizuhira & Futaesaku, 1972 Tilney et al 1973 Burton et al 1975 Fujiwara & Tilney 1975)

A mixture of tannic acid with the aldehyde group has a higher sensitivity to proteins than has the aldehyde group alone Most of the proteins precipitate at an acidic pH range (Mizuhira & Futaesaku 1972) In the current experiment, the final pH of the fixatives was adjusted to 6.0 in order to obtain a satisfactory fixation of the specimens The negative stain-like effect of the tannic acid fixation revealed the fine filamentous structure of the rootlets in greater detail than did the ordinary fixation method. This fixation effect suggests the existence of biochemical multi-phases in the structue of the rootlet.

Since the introduction of electron microscopy it has become clear that cytoplasma possess at least four types of submicroscopic fibril including: microtubules (180–250 Å in diameter) so-called tonofilaments (80–100 Å in diameter) myosin filaments and microfilaments (40–70 Å in diameter) Microfilaments are seen not only in muscle cells but also in all other cells.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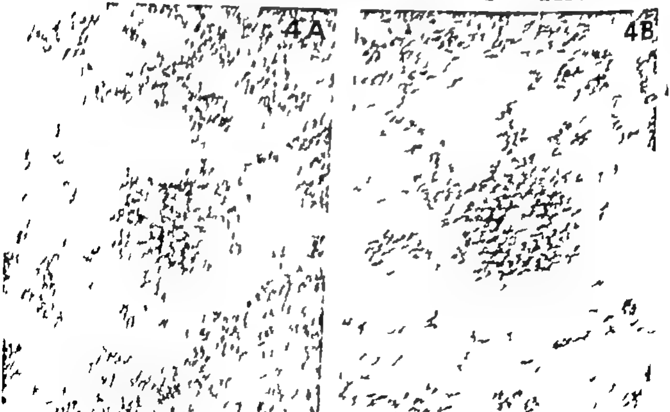
4A
4B

Fig 5 Much higher magnification of the filamentous structure. Hexagonal pattern is formed by protofilaments in rootlet (*H*) 1 000 000.

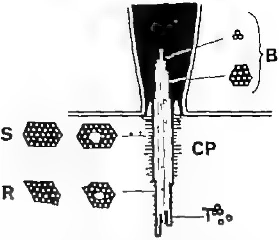

Fig 7 Schematic drawing of fibrillar structure in the basal portion of hair and in rootlet by tannic acid fixation method *B* basal portion of hair *S* cuticular surface *R* rootlet *T* terminal of rootlet *CP* cuticular plat

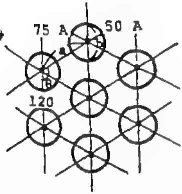

Fig 6 Schematic drawing of hexagonal pattern in Fig 5

It has been proved by many investigators that microfilaments are composed of actin (Ishikawa et al 1969 Lazarides & Weber 1974 Goldman et al 1975 Edds 1977) It has also been found that the stereocilia filaments of the inner ear sensory cells are also composed of actin (Flock & Cheung 1977) Protofilaments in this study are considered to be modified microfilaments because they are more regularly and straightly arranged than are ordinary microfilaments The diameter of each modified microfilament accords with that of G-actin (50 Å)

In examining the mechanical properties and physical functions of the microfilaments it is possible that the microfilaments can mechanically sustain each hair at the base. The parallel filaments and the finer chain-like filaments between them have a probable elasticity to bend by force of physical displacement and also enable the hair to stand erect in the resting state Flock & Murray have already investigated the mechanical properties of sensory hairs in the crista ampullaris of the semicircular canal and observed that the hairs appear quite stiff pivoting around their base (1977) The elasticity of the modified microfilaments is suspected to play a part in the pivoting movement. The bending force induced by displacement of hair may secondarily produce A-scale strains of the crystalline-like structure of the cross-sectioned rootlet. As a working hypothesis one could assume that the paracrystalline structure generates a signal by the small strains in response to auditory stimulus It is of interest that rootlets may act as a part of the energy conversion system The author

Fig 3 Cross-sectioned rootlets at the level of cuticular plate of inner hair cell Varied-sized solid and tubular shaped rootlets are seen Note the lighter zone around each rootlet Number of protofilaments can be counted in an electron-dense substance of each rootlet 120 000

Fig 4 High magnification of rootlet of inner hair cell (A) Solid-shaped rootlet (B) Tubular-shaped rootlet. Finer filament chain the protofilaments to one another Small branches radiate from the background substance 400 000

thinks that the profilament provides elasticity at the basal portion of the hair

ZUSAMMENFASSUNG

Die Haarwurzeln der akustischen Sinneszellen beim Meerschweinchen wurden mit dem Elektronenmikroskop druch die Methode der Tanninsäure-Fixierung studiert. Bei der Methode der Tanninsäure Fixierung zeigt sich das Bild der feineren Faserstruktur in größerem Detail auf als mit der Methode der Glutar-Osmium-Fixierung Die Wurzel hatte sich dabei aus 50-Å Protofilamenten im Durchmesser und aus einer elektrondichten Substanz im Bild zusammengesetzt. Die Protofilamente in jeder Wurzel waren einander gleichlaufend und tief in die Kutikula eingedrungen Im Querschnitt jeder Wurzel war das fundamentale Aufstellungsmuster der Protofilamente regelmäßig hexagonal Daher wurden die Protofilamente als modifizierte Mikrofilamente betrachtet.

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M Itoh M D
Dept of Otorhinolaryngology
Faculty of Medicine
Kyushu University
Fukuoka 812
Japan

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EARLY STAGES OF MYELINATION IN THE SPIRAL GANGLION CELLS OF THE KITTEN DURING DEVELOPMENT

R Romand, M R. Romand Ch Mulle and R Marty

From Laboratoire d Neurophysiologie Université Montpellier II France

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Abstract Myelinated cell bodies of spiral ganglion in kittens during development can first be distinguished by light microscopic observation at the end of the first postnatal week By means of electron microscopic observation, the first signs of myelinated perikarya can be observed around the time of birth Myelination is preceded by the ensheathment of the ganglion cell body by two or three layers of Schwann cell cytoplasmic processes At birth, the first sign of myelination is visible at the basal part of the cochlea with thin thickenings which contain two or three major dense lines

At this stage these thickenings cover only small perikaryal area Later on, the thickness and the number of thickenings increase and cover the major part of the cell body The onset of myelination of the processes seems to precede the perikaryal myelination

A large number of publications have been devoted to the cochlear receptors and peripheral processes of ganglion cell maturation in mammals including those by Kikuchi & Hilding (1965), Nakai (1970) Pujol & Marty (1970) and Pujol et al (1978) However only a few studies have been devoted entirely to the spiral ganglion cells and their central processes (Trevin 1962 Bruckman 1972 Thorn & Arnold 1976 Romand et al 1976) The cell bodies of the main population of spiral ganglion cells in adult cochlea (Spoendlin 1972) are peculiar because they are covered by several myelin layers which may be either compact or loose Observations by means of electron microscopy by Rosenbluth & Palay (1961) in goldfish by Rosenbluth (1962) in rats, by Kellerhal et al (1967) in various mammals, and by Adamo & Daigneault (1973) in cats have shown different patterns of myelin sheath

Our investigation presents some observations on the early stages of myelin sheath formation of the kitten s spiral ganglion cells in order to provide an extended basis of comparison for future experimental studies

MATERIAL AND METHODS

In order to study the spiral lamina ? fetuses 24 kittens ranging from birth to one month and 2 adult cats were investigated. After an in vivo perfusion with a mixed solution of glutaraldehyde-paraformaldehyde the spiral lamina was removed and post-fixed in a 2% osmium tetroxyde solution After dehydration the tissue was embedded in Araldite. Semithin sections were stained with toluidine blue for light microscope observation. Thin sections were stained with uranyl-acetate and lead citrate and observed with a Jeol 100 B electron microscope

RESULTS

Light microscopic observations

Early signs of cell myelination can be seen at the basal part of the cochlea a few days after birth Just at birth it is very difficult to differentiate a myelinated cell body from an unmyelinated perikaryon even by phase contrast observation, because some spiral ganglion cells are surrounded by dark glial processes which make the cell appear myelinated (Figs 1–) The first signs of myelination ap-

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thinks that the profilament provides elasticity at the basal portion of the hair

ZUSAMMENFASSUNG

Die Haarwurzeln der akustischen Sinneszellen beim Meerschweinchen wurden mit dem Elektronenmikroskop druch die Methode der Tanninsäure Fixierung studiert. Bei der Methode der Tanninsaure Fixierung zeigt sich das Bild der feineren Faserstruktur in größerem Detail auf als mit der Methode der Glutar-Osmium-Fixierung Die Wurzel hatte sich dabei aus 50-Å Protofilamenten im Durchmesser und aus einer elektrondichten Substanz im Bild zusammengesetzt. Die Protofilamente in jeder Wurzel waren einander gleichlaufend und tief in die Kutikula eingedrungen Im Querschnitt jeder Wurzel war das fundamentale Aufstellungsmuster der Protofilamente regelmäßig hexagonal Daher wurden die Protofilamente als modifizierte Mikrofilamente betrachtet.

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M Itoh M.D
Dept of Otorhinolaryngology
Faculty of Medicin
Kyu h Uni ersity
Fukuoka 812
Japan

pear after the first week, as can be seen on a few cells from the basal part of the cochlea. However it is sometimes difficult to differentiate the myelin sheath from the dark glial processes

The difference in myelination between the perikaryon and the processes is very striking as observed at birth in Fig 1 where myelination of the central process is underway while no myelin sheath is visible on the cell body under light microscopic observation At the basal part of the cochlea there are myelinated fibres with unmyelinated cell bodies (Figs. 1–2). From the age of one month up to the adult stage the myelin sheath is clearly visible around the perikaryon (Fig. 3) though the difference in myelination is still visible between the processes and the perikaryon The myelin sheath of the processes is thicker than that of the cell body

Electron microscopic observations

Myelination is preceded by profound glial activity which is seen mainly by means of a Schwann cell layer around the cell body and extends to the peripheral and central processes (Fig 4) Later on one can observe one or two additional cytoplasmic layers The first sign of myelination—using the criterion for myelination in spiral ganglion cells developed by Kellerhals et al (1967)—is produced by the fusion of the cytoplasmic surfaces of two Schwann cell membranes in order to produce a major dense line This appears only on some parts of the perikaryal surface as a thin thickening. It is usually difficult to find the first thickening of Schwann cell membranes most often the first two myelin layers are the early sign of myelination that is highly visible Unlike myelination of axonal internodes where the formation of myelin is uniform all around the axoplasm for the perikaryal internode myelination is rather different

The earliest stage of myelination that appears around birth is characterized by a few short black thickenings of various lengths around the cell body when the cell is observed at low magnification (Fig 5) During the subsequent days other thickenings appear around the cell body At higher magnification each thickening is separated from the others by unfused Schwann cell membranes The thickenings are formed of two or more major dense lines (Fig 6)

Later during the postnatal development the thickenings lengthen and proliferate and the number of lamellae in each thickening increases The perikaryon is almost completely surrounded by a myelin sheath although some parts still lack compact myelin

At 10 days all spiral ganglion cells from the basal part to the second turn of the cochlea, appear to be more or less myelinated Even at the same location spiral ganglion cells are at various stages of myelination. Some have only a few thickenings around their perikarya, while others along with their proximal processes up to the first node of Ranvier are almost completely surrounded by myelin sheaths As maturation continues it becomes characterized by an increasing number of lamellae and a decrease in the area where the compact myelin sheath does not appear (Fig 7) In the area where the compact myelin sheath does not exist it is possible at high magnification to observe well separated Schwann cell membranes or some major dense lines loosely packed (Fig. 8)

DISCUSSION

The first sign of myelination using light microscopic observation may be visible at the end of

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Fig 1 Spiral ganglion cell from newborn kitten Note the central process (*CP*) where the myelin sheath can be seen after the first node of Ranvier (arrow). No myelin sheath is visible on the perikaryon Basal part of the cochlea as for the subsequent figures Semithin section 1000

Fig 2 Spiral ganglion cells and Schwann cells (*white arrow*) from newborn kitten Glial processes around the perikarya are marked by black arrow Semithin section, 1400

Fig 3 On cochlea of 1-month-old kitten, the myelin the spiral ganglion cell

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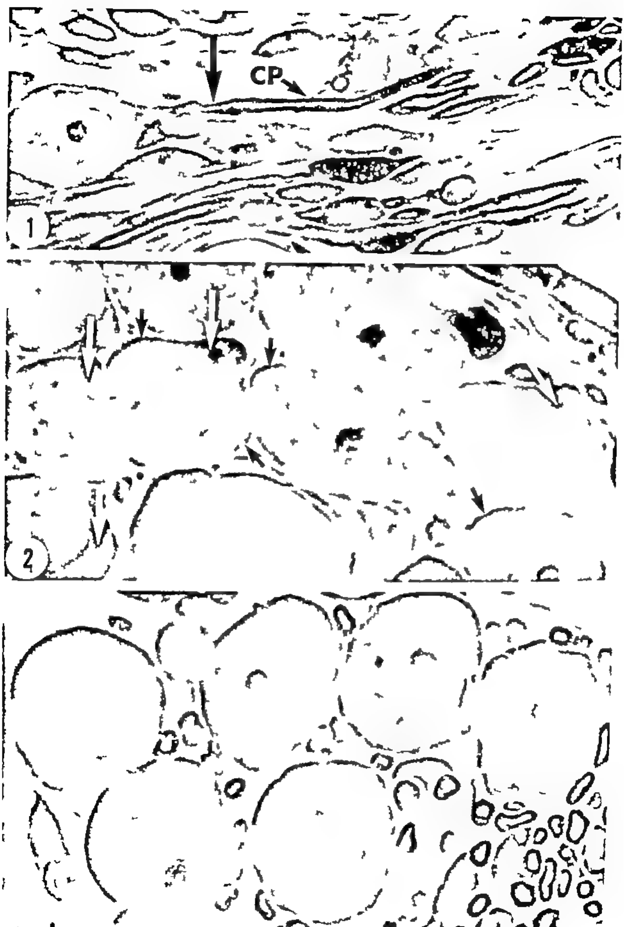

CP
1
2
3

the first postnatal week, although Pujol & Marty (1970) reported myelinated spiral ganglion cells at birth. At this stage of development, it seems very difficult to differentiate the few thin thickenings that can be observed by electron microscopy from glial processes by light microscopic observation. As pointed out by Kellerhals et al (1967) concerning the myelin sheath of spiral ganglion it is better to interpret light microscopic observations concerning the presence or absence of the myelin sheath in correlation with electron microscopic observations Using this technique it is possible to observe the first sign of myelination which starts at the basal part of the cochlea at birth with one or two thin thickenings around the perikaryon.

The beginning of myelination of the spiral ganglion cell bodies seems to start a few days after their peripheral and central processes This can be seen by means of indirect and direct evidence

A previous study of the development of the central processes (Romand et al 1976) has shown that myelinated auditory fibres are already present at least 6 days before birth At this period the ganglion cell bodies are still densely packed and do not show any sign of myelination Another example is given in Fig 4 in a newborn kitten where a well myelinated fibre is close to an unmyelinated cell body that represents the most numerous cell population at this stage Another fact tends to confirm the above observations On a few cells it was not possible to see any signs of myelination on the perikaryal internode after electron microscopic observation while the axonal internode was relatively well myelinated

A tentative schema can be drawn concerning the myelination events which start after the ensheathment of the perikaryon by two or three layers of Schwann cell cytoplasm and might correspond to the promyelin stage in peripheral nerve fibres. At this stage the cytoplasmic layers do not seem to be very different from those of the loose myelin of the adult cat (Adamo & Daigneault, 1973) Later after the first appearance of compact myelin as revealed by a thickening the area covered by the loose myelin tends to decrease and to be replaced by the compact myelin that surrounds all the perikaryal surface in some cells in the adults However at this stage it is still possible to see the two types of myelin observed by Kellerhals et al (1967) in the guinea pig and by Adamo & Daigneault (1973) in the cat In some segments ensheathment may be composed of a combination of layers of loose and compact myelin while other segments of the same cell may be covered merely by loose or compact myelin

In the guinea pig Thorn & Arnold (1976) described a myelin sheath formation which is quite similar in the early stages of development However an intermediate stage (a semi compact myelin) between the loose and the compact myelin seems to be more frequent than in the cat in the early stage of myelin sheath formation In the cat the compact myelin occurs more frequently although some semi-compact myelin can be seen around the perikaryon

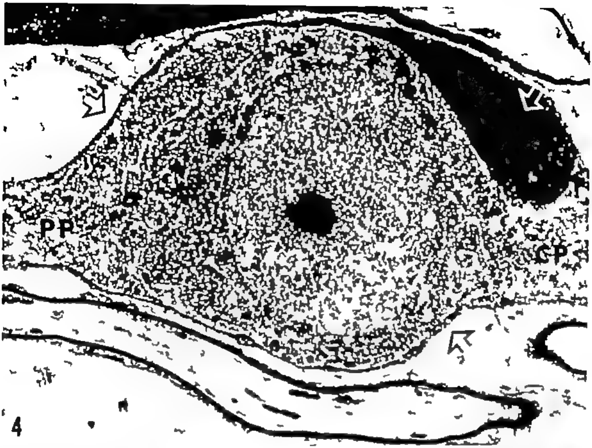
Fig 4 Electron micrograph of an unmyelinated spiral ganglion neuron from newborn kitten The Schwann cell (white arrow) with processes (black arrows) ensheath the spiral ganglion cell body and part of its peripheral process (*PP*) and its central process (*CP*). 5600

Fig 5 Early sign of myelination seen on the cell body of spiral ganglion neuron from new born kitten. The thickenings produced by myelin sheath are indicated by arrows 4600

Fig 6 High magnification of thickening that is produced in this case by two major dense lines. Basement mem- ganglion cell cytoplasm

ZUSAMMENFASSUNG

Beim Kätzchen können die ersten myelinisierten Perikarya lichtmikroskopisch gegen das Ende der ersten Woche beobachtet werden. Elektronenmikroskopisch können die ersten Anzeichen der Myelinisierung so um die Geburtszeit gesehen werden. Vor der Myelinisierung des Perikaryons beginnt die Wickelung von zwei oder drei cytoplasmatische Fortsätzen aus der Schwannschen Zelle. Bei der Geburt ist das erste Zeichen der Myelinisierung am basalen Teil der Cochlea sichtbar und durch feine Verdickungen charakterisiert, die zwei oder drei „major dense lines" enthalten. Auf diesem Stadium sind diese


PP
CP
4


5


B
M
C
6

Verdickungen nur auf einer kleinen Fläche des Perikaryons sichtbar. Später nehmen sie an Dicke und Zahl zu und bedecken den größten Teil des Perikaryons. Der Beginn der Myelinisierung der Zellenfortsätze scheint der des Perikaryons vorauszugehen.

RÉSUMÉ

Chez le chaton, les premiers corps cellulaires myélinisés du ganglion spiral peuvent être observés en microscopie optique vers la fin de la première semaine. Grâce à l'observation en microscopie électronique, les premiers signes de myélinisation peuvent être vus aux alentours de la naissance. La myélinisation du corps cellulaire est précédée par l'enroulement de 2 ou 3 expansions cytoplasmiques en provenance de la cellule de Schwann. A la naissance, le premier signe de myélinisation est visible à la partie basale de la cochlée et se caractérise par de fins épaississements qui contiennent 2 ou 3 lignes majeures. A ce stade, ces épaississements sont visibles seulement sur une petite surface. Plus tard, leurs épaisseurs et leurs nombres augmentent et couvrent la plus grande partie du corps cellulaire. Le début de la myélinisation des expansions cellulaires semble précéder celle du soma.

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R. Romand, Ph.D.
Laboratoire de Neurophysiologie
Université de Montpellier II
Place Eugène Bataillon
F 34060 Montpellier Cedex
France

Fig 7 Ganglion cell from 1-month-old kitten Some parts of the perikaryon lack of compact myel n sheath (*arrows*) ×6900

Fig 8 High magnification of myelin sheaths from two different myelinated ganglion cells (1-month-old kitten) The first cell (*1*) presents at this spot a loose or semi-comp myelin sheath The second cell (*2*) ha a compact my sheath The two sheaths are separated by glial proces (*G*) and th intercellular space (*I*) ×37 000

Table I *Summary of patients with Malignant External Otitis*

Patient	Age/Sex	Diabetic therapy	Days of hospitalisation	Treatment	Neurological symptoms
1 H R.	67/♀	Insulin	108	Rad. Mastoidectomy parotidectomy Carbenicillin, gentamicin, tobramycin colistin, cefalotin	Facial nerve paralysis
2 G R.	75/♀	Chlorpropamide	44	Gentamicin, carbenicillin co-Trimoxazole, colistin	
3 K M	85/♂	Diet	11	Gentamicin	
4 S Y	76/♂	Diet	20	Polypectomy colistin, co-Trimoxazole	

MATERIALS AND METHODS

Delayed hypersensitivity reaction to recall antigens was tested by intradermal injection of 0.1 ml of the following reagents PPD (Parke Davis Detroit, Mich) 5 TU and 250 TU SK SD Varidase (lederle Laboratories Pearl River N Y) 2.5 U and 10 U and Mumps skin test antigen (Eli Lilly & Co Indianapolis Ind. USA) Reactions were read at 48 hours and graded as negative when induration was less than 5 mm

Peripheral blood lymphocytes transformation *in vitro* was tested with the following reagents PHA P (Difco Laboratories Detroit, Mich USA) 3 μg/ml and 20 μg/ml ConA (Miles-Yeda Ltd Israel) 1 μg/ml and 5 μg/ml and PWM (Gibco Grand Island N Y) 1 μg/ml and 5 μg/ml Ficoll-Hypaque separated lymphocytes were adjusted to a concentration of 0 5 10^6 cells/ml in RPMI 1640 medium (Gibco Grand Island N Y) supplemented with 100 U/ml penicillin 100 μg/ml streptomycin 5% heat inactivated fetal calf serum (Gibco) and with $5 \times 10^{-}$ M mercaptoethanol (Eastman Kodak Co Rochester N Y) Samples of 0 ml (10^6 lymphocytes) were placed in sterile microtitre plates (Falcon 3040) with or without mitogens Cultures were performed in triplicate for 72 hours in a humidified atmosphere containing 5% CO at 37°C Four hours before termination 0 01 ml containing μCi of tritiated thymidine (Israel AEC Negev Israel specific activity 5–10 Ci/mmol) was added to each of the wells. The cultured cells were collected onto fibre glass filters (GF/C Whatman) using an automated multiple cell harvester Samples were counted in a β-spectrometer (Packard Instruments) using Bray s solution as a scintillation fluid Results are expressed as c p m ± S E.

Serum immunoglobulins (IgG IgM IgA) as well as complement components C3 and C4 were determined by single radial diffusion in agar with Hoechst plates Total hemolytic activity of complement was measured by lysis of sensitized erythrocytes incorporated into agarose-containing immunodiffusion plates (Gewurz & Suyehira, 1976) Activity was expressed as a percentage of the lysis caused by standard guinea pig complement (Behringwerke AG Germany)

Peripheral blood lymphoid cells were evaluated for surface cell markers by the sheep erythrocyte rosetting assay by fluoresceinated heat aggregated human Ig and by fluoresceinated F(ab) fragments of rabbit anti-human IgG IgM IgA and IgD (Bentwich & Kunkel 1973) The number of monocytes was determinated by latex ingestion and the function of neutrophils was evaluated by the NTB (nitroblue tetrazolium) assay (Park et al 1968)

RESULTS

All 4 patients demonstrated impaired cellular immunity as shown by *in vivo* and *in vitro* tests Delayed hypersensitivity skin tests to PPD SK SD and Mumps antigens were all

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IMPAIRMENT OF CELLULAR IMMUNITY IN PATIENTS WITH MALIGNANT EXTERNAL OTITIS

I Yust [1] C Radiano [2] B Tartakovsky [3] A Tanay [1]
E Shanon [2] and S Segal [3]

From the Departments of Medicine B' and [2]Otolaryngology Ichilov Hospital and Tel-Aviv University Medical School and the Department of Cell Biology The Weizmann Institute of Science Rehovot Israel

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Abstract Immunological studies were performed on 4 elderly diabetic patients with Malignant External Otitis caused by *Pseudomonas aeruginosa* Impairment of cellular immunity was found Skin tests for delayed hyper sensitivity to PPD SK SD and Mumps antigen were negative and stimulation rates of peripheral blood lymphocytes by PHA Con A and PWM were depressed in all 4 patients Serum immunoglobulins and complement levels were normal exept in one case in which paraprotein IgG was found Peripheral blood lymphoid cell markers and the neutrophil nitroblue tetrazolium (NBT) test were within normal limits in the 2 patients tested These results indicate that cellular immune deficiency predisposes to the development of Malignant External Otitis in elderly diabetic patients.

The entity of Malignant External Otitis (MEO) was first described by Chandler in 1968 It is a severe form of external otitis occurring almost exclusively in elderly diabetic patients The typical features are severe edema and tenderness of the external canal a purulent discharge from which *Pseudomonas aeruginosa* can be cultured the presence of granulation tissue in the floor of the canal at the bony cartilaginous junction and an intact tympanic membrane If not treated vigorously the condition can lead to serious complications such as extension to adjacent cartilage bone nerves and soft tissues resulting in osteomyelitis of the skull multiple cranial nerve palsies and death Of the 77 patients described in the English literature until 1977 27 (35 %) died of the disease (Meyrhoff et al 1977)

The reason for the low resistance of these patients to pseudomonas infection is not clear although some investigators have suggested the existence of an immunological disturbance in these patients (Meyrhoff et al 1977 Zaky et al 1976 Chandler 1977) In this communication we bring evidence for impairment of cellular immunity in 4 patients with Malignant External Otitis

PATIENTS

Four patients were studied (Table I) All had the typical features of unilateral Malignant External Otitis They were elderly diabetics and had been treated unsuccessfully for several weeks outside the hospital with topical and systemic antibiotics and by cleansing of the affected aer On admission to the hospital all had a suppurative discharge from which *Pseudomonas aeruginosa* was isolated and granulation tissue in the external canal Treatment consisted of massive systemic (i v) and local antibiotics and in one case mastoidectomy and parotidectomy were performed Results of routine laboratory tests including white and red blood cell counts urea creatinine and electrolytes were within normal limits The diabetes was well controlled before and during the disease with insulin in one patient with chlorpropamide in one and with diet in 2 only All 4 patients survived the MEO and remained well between 7 to 18 months after recovery

Table II *Immunoglobulin and complement levels in patients with Malignant External Otitis*

Patient	IgG (mg/%)	IgM (mg/%)	IgA (mg/%)	C3 (mg/%)	C4 (mg/%)	Hemolytic activity (%)
1 H R	1 900	125	225	118	5	43
2 O R	950	155	520	105	60	53
3 K M	1 980	185	465	116	66	70
4 S Y	2 320	6_	120	112	45	65
Normal range	700–1 900	45–180	90–450	55–120	20–50	22 55

Paraprotein IgG λ

positive (7 mm of induration) but PPD and SK SD skin tests remained negative Except for an improvement in the response to PHA, responses of lymphocytes to Con-A and PWM in vitro did not improve on repeated testing (Fig.) Levamisole was tolerated well except for development of eosinophilia (up to 50% peripheral blood leukocytes) which subsided gradually after discontinuation of the drug.

Immunoglobulin levels were within the normal or above the normal range (Table II) In one patient (No 4) an IgG paraprotein was found Serum C3 C4 and hemolytic activity levels were normal (Table II) In 2 patients (Nos 1 and 2) quantitation of peripheral blood T and B cells and monocytes was normal (Table III) The function of peripheral blood phagocytes was measured by the NBT test and was also found normal

DISCUSSION

Pseudomona aeruginosa is an ubiquitous opportunistic Gram negative organism found frequently in external otitis following trauma or swimming (Wright & Alexander 1974) but causes Malignant External Otitis mainly in elderly diabetic patients It is not clear which features of the diabetic patient predispose to this severe infection It has been suggested that disease of the small blood vessels is an important factor and that the pathologic changes in the ears and temporal bones of patients with MEO constitute the otologic equivalent of diabetic gangrene of the limbs (Evans & Richards 1973 Zaky et al 1976) However this does not explain why so few patients with diabetes mellitus develop MEO Some clinical features in MEO suggest immunological impairment. The few MEO patients reported who were not diabetics suffered from diseases associated with immunosuppression, such as leukemia and granulocytopenia (Chandler 1977 Meyrhoff et al 1977) Moreover the excessive granulomatous response to the otologic pseudomonas infection resembles the granulomatous disease of childhood caused by a primary defect in the intracellular digestion of bacteria phagocytosed by granulocytes (Jonston & Baehner 1971) This and the fact that severe pseudomonas infections with sepsis occur mainly

Table III *P riph ral blood l mphoid and phagocytic cells markers*

Patient	% E Rosette (T cells)	% Ig cells (B cell)				% Ig aggregates	% latex positive
		IgG	IgM	IgA	IgD		
1 H R	94	3 0	7 1	5	6.1	28.8	28 8
2 O R	87	8 6	36 6	8	15 0	10 0	20 0
Normal ± S D	80±4	8±4	10 4	±1	8 3	18_7	20±5

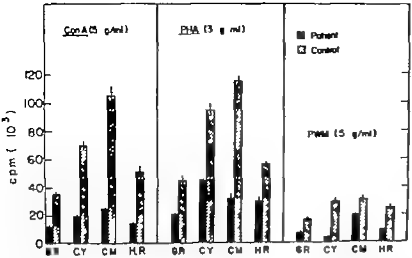

Fig 1 Responses to mitogens in 4 MEO patients and healthy controls. Values shown represent absolute ± s.m.

negative except in patient No 3 to PPD 250 TU Stimulation rates of lymphocytes with PHA Con-A and PWM were significantly lower in all patients with MEO than in normal controls age and sex matched (Fig 1) In one patient (No 1) skin tests were repeated three times during the course of the disease and were consistently negative In addition stimulation rates of this patient s lymphocytes with mitogens were depressed during the disease and remained so for as long as 10 months after recovery (Fig 2) In each experiment the patient s lymphocytes were tested together with lymphocytes from a different normal control and the results are expressed as percentages of the normal control values The stimulation rates of the patient s lymphocytes with Con A and PWM were lower than those of normal controls the response to PHA was on some occasions also depressed but to a lesser extent To improve this patient s cellular immunity levamisole treatment 50 mg three times a day twice weekly was started on the 45th day of hospitalization and was continued for 9 weeks After this treatment delayed hypersensitivity skin tests to Mumps became

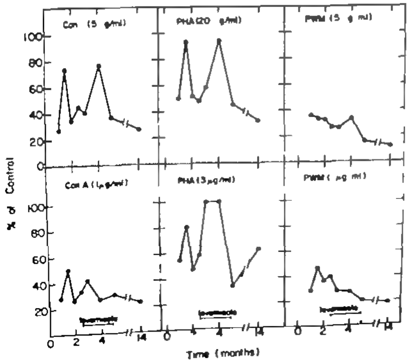

Fig 2 The responses to mitogens in one MEO patient (H R.) followed up for 14 months. Values given represent percentages of normal control stimulations.

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in compromised hosts (Margaretten et al 1961) stimulated us to evaluate the immunological status of MEO patients

Our study clearly demonstrated impairment of cellular immunity in patients with MEO Skin tests for delayed hypersensitivity with PPD SK SD and Mumps antigens were negative *In vitro* PHA Con-A and PWM induced lymphocyte transformation was also depressed though the number of populations of T and B lymphocytes and monocytes was normal This indicates a functional cellular defect associated with MEO The immunological disturbance in MEO patients appears to be primary and not secondary to the severe pseudomonas infection since in one patient it had not improved even 10 months after complete recovery (Fig 2) A similar picture of normal B and T values in the peripheral blood in the face of severe invalidation of the immune system has been found in other patients suffering from a variety of autoimmune disorders (Glinski et al 1976) The differences observed in the degree of impairment of response to PHA as compared with Con-A and PWM as well as the relative recovery of the PHA response (but not the Con-A and PWM response) following treatment with levamisole in one patient (H R) could be expected Recent data show that the response to PHA may be mediated by a different T cell subset and may be subject to different control mechanisms than those governing the responses induced by Con A and PWM (horwitz & Garrett 1977) This indicates that results obtained by the use of PHA alone for *in vitro* diagnosis of immunodeficiencies may be misleading

All our patients with MEO were elderly diabetics Hence the observed immunosuppression may be the result of old age and diabetes contributing to a functional immunodeficiency though this is not necessarily the case Although several investigators have shown impairment of lymphocyte responses to PHA in old people (Hallgren et al 1973) and in diabetics (Delespesse et al 1974) others have demonstrated normal lymphocyte mediated reactions in the aged (Ben-Zwi et al 1977 Portaro et al 1978) and norma PHA induced lymphocyte transformation i well controlled diabetics (MacCuish et al 1974) PHA induced lymphocyte transforma tion (Ragab et al 1972) as well as granulo cyte chemotaxis (Mowat & Baum 1971) phagocytosis (Bagdade et al 197?) and micro bicidal function (Tan et al 1975) were de pressed only in poorly controlled diabetics It is therefore important to stress that the dia betes of our 4 patients was well controlle prior to and during the pseudomonas infection

Our findings suggest that cellular immun deficiency is involved in the development o Malignant External Otitis to substantiate the conclusion additional elderly diabetic patient with and without MEO should be investigated

ACKNOWLEDGMENTS

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ZUSAMMENFASSUNG

Bei 4 alteren diabetischen Patienten mit durch Pseudo monas Aeruginosa verursachter maligner Otitis Extern wurden verschiedene immunologische Untersuchunge durchgefuhrt Es fand sich eine Beeintrachtigung de zellularen Immunitat Hautproben zur Untersuchung de verspateten Uberempfindlichkeit der PPD- SK-SD- un Masern-Antigene waren alle negat ve Die Stimulation raten der peripheren Blut Lymphozyten durch PHA Con A und PWM waren bei allen 4 Patienten herabgesetzt Die Immunoglobuline und der Komplement Spiegel waren normal bis auf einen Patienten mit Paraprote vom Typ IgG Bei zwei Patienten wurden die lymphoide Zellmarker und der neutrophile N troblau-Tetrazolium Test (NTB) tudiert und waren im Normbereich Diese Resultate weisen darauf hin daß altere Diabetiker mi herabgesetzter zellularer Immunitat zur malignen Otitis externa pradisponiert sind

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I Y st M.D
Ichilov Hospital
W Esmena St 6
Tel-A
Israel

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QUANTIFICATION OF TRACKING EYE MOVEMENTS IN NORMAL SUBJECTS

Lucyna Schalén

Department of Otorhinolaryngology University Hospital Lund S eden

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Abstract Voluntary tracking eye movements were ana lysed in 20 normal subjects whose gaze was fixed on a visual target moving at six different constant velocities between 10° s and 60° s Tracking ability was quantified according to four parameters. The mean value and dispersion of each parameter at each velocity were deter mined *The maximum velocity gain of smooth pursuit* was on a erage 0 98–0 75 gradually diminishing with increasing target velocities of 10–60° s *Amplitud of smooth pursuit* decreased gradually and was replaced by superimposed saccades at increasing target velocities *Saccades* with amplitudes of 3–10° were present at all target velocities while those greater than 10° occurred mostly at target velocities above 30° s *Square waves* were rare but equally frequent at all target velocities and seemed to occur randomly during tracking eye move ments.

An additional group of 9 subjects was investigated twice Mean values of maximum velocity gain of amplitude of smooth pursuit and of frequency of superimposed saccades were higher on the second occasion probably reflecting the effect of learning

Co-operation and interaction of the smooth pursuit and the saccadic subsystems to produce the voluntary tracking were discussed

During voluntary tracking eye movements the image of a visual target is kept on the fovea despite movements of the body the head or of the target The accuracy of tracking has been analysed mostly with respect to velocity gain i e the ratio of eye velocity/velocity of the target It has been shown that the gain is close to 1 00 at low target velocities (Baloh et al 1976 Kowler et al 1978 Sharpe et al 1979) However the amplitude of the pursuit eye movements in relation to the movement of the target has not received much attention Few efforts have been made to quantify the superimposed saccades i e those rapid eye movements which fix the fovea again on the target in case of a foveal image slip during tracking Although the phenomenon has been studied previously (Westheimer 1954 Brown 1972) quantitative analyses of saccadic corrections with respect to different target velocities have rarely been performed and in those instances the analysis was concentrated on saccadic frequencies but not on saccadic amplitudes (Sharpe & Sylvester 1978) or else the testing applied to only one target velocity (Hartje et al 1978)

Square waves interfering with smooth pursuit eye movements have mostly been interpreted as indicative of a pathological condition (Dell Osso et al 1975 Feldon & Langston 1977) or as resulting from the lack of visual control of eye movements (Kornhuber 1974) Only a qualitative description of square waves during fixation on a moving target in healthy subjects has so far been published (Jung & Kornhuber 1974)

The aim of the present investigation was to analyse the variation in voluntary tracking eye movements at different target velocities with special reference to velocity gain smooth pursuit amplitude saccadic corrections and square waves

MATERIAL AND METHODS

Tracking movements of the eyes were studied in 20 volunteers 10 males and 10 females of mean age 49 9 (range 22–70) years

An additional group of 9 volunteers was examined on two separate occasions in order to study intra-individual variation This group

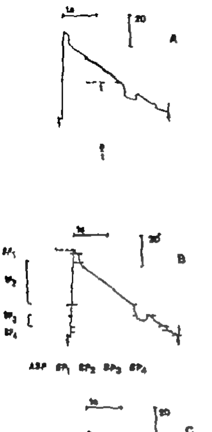


Fig. 1. Illustration of EOG recordings of voluntary tracking eye movement in a normal subject at target velocity of 10° s⁻¹. Arrows indicate extreme positions of the visual target. A saccade to the right brings the eyes on the target. Subsequent tracking to the left consists of smooth pursuit (SP), superimposed saccades (SS) and square waves (SW). A. Maximum velocity of smooth pursuit. B. Amplitude of smooth pursuit (ASP). C. Superimposed saccades and square waves.

consisted of 3 women and 6 men aged 22–34 (mean 25.3) years.

The subjects had no previous history of neurological or otological disturbances and no signs of pathology were found at the otoneurological examination (Henriksson et al. 1972). No drugs were allowed within 48 hours of the test (Rashbass 1961).

The visual target was a spot of light 30 mm in diameter projected on a screen 160 cm from the subject. The target was driven in predictable ramps of 60° amplitude at the six velocities of 10° 20° 30° 40° 50° 60° s⁻¹. The target could travel in both directions. As soon as the target disappeared in one direction a new one became visible on the opposite edge of the screen. Extreme positions of the target on the screen were detected by phototransistors and recorded.

The subject was seated in a chair with the head fixed by occipital supports. The movements of the eyes could be monitored during the test with an infrared-operated TV camera. The subject was instructed to pay attention to the target and to follow it as accurately as possible avoiding blinking. Verbal contact was maintained throughout the procedure in order to keep the attention on the visual task.

Each test procedure was started by a tracking to the right at the lowest target velocity. Five consecutive trackings were recorded at each velocity. The procedure was subsequently repeated to the left.

Eye movements were recorded with a d.c. electro-oculographic technique using commercial electrodes and jelly (Medicotest®). Horizontal eye movements were recorded binocularly and monocularly in the vertical plane (Henriksson et al. 1972, Gay et al. 1974). Recordings were displayed by a jet-ink writer (Mingograph M-81, Siemens-Elema, Stockholm, Sweden) with an upper cut-o[ff] frequency of 15 Hz. The recording paper wa[s] fed at a velocity of 25 mm s⁻¹ allowing a[n] accuracy of time events of 40 ms correspon[d]ing to 1 mm in the recording. The recordin[g] permitted the resolution of eye position to a[n] accuracy of 1° corresponding to 1 mm on th[e] recording. Calibration of eye movements wa[s] taken from voluntary saccades 20° and 60° [to] the left and to the right.

The following parameters were evaluated:

The maximum velocity gain of smooth pu[r]suit eye movement (*MVSP gain*) was calcul[at]ed from the ratio maximum velocity of smoo[th]

Table I *Tracking eye movements in 20 normal subjects*

Means of the individual means and standard deviations (in parentheses) of the tested parameters during tracking to the right at different target velocities

	Target velocity (deg s)					
	10	20	30	40	50	60
Maximal velocity gain	0 95 (0 10)	0 98 (0 12)	0 94 (0 1?)	0 92 (0 15)	0.86 (0 14)	0.75 (0 11)
Total tracking amplitude in deg	60 0 (7 7)	57 6 (8.9)	53 0 (11.2)	52.3 (9 6)	46 3 (12 4)	41 6 (11 3)
Amplitude of smooth pursuit in deg.	52.8 (10 0)	51 1 (9 7)	45.8 (11 4)	44.5 (9 9)	36 6 (10.5)	[illegible] (8 3)
Superimposed saccades frequency in 60s of testing						
3–10°	*6 (5)*	*11 (11)*	*20 (17)*	*33 (23)*	*35 (28)*	34 (23)
11–20°	0	3 (5)	4 (4)	9 (14)	16 (14)	18 (20)
21 –>	0	0	1 (4)	3 (5)	4 (8)	11 (20)
Square waves frequency in 60 s of testing	8 (10)	5 (3)	4 (8)	1 (3)	6 (6)	[illegible] (8)

pursuit eye movement/target velocity The maximum velocity of smooth pursuit eye movement (*MVSP*) was registered after an initial velocity adaptation period of 500 ms and calculated from a part of the smooth pursuit eye movement which lasted at least 250 ms i e the shortest time for the visual perception of the movement (Yarbus 1967) (Fig 1*a*)

The amplitude of smooth pursuit eye movement (*ASP*) was defined as the sum of all smooth pursuit eye movements in each tracking after excluding saccades and square waves (Fig 1*b*)

The total tracking amplitude (TA) was equal to the amplitude of returning saccades

The superimposed saccades (*SS*) were classified according to the amplitudes into three groups 3–10° 11–20 and 21 or more (Fig 1*c*) SS smaller than 3 were not included

The square waves (*SW*) were defined as two saccades directed counter to each other and having an interval of relative standstill (Fig. 1*c*)

Measurements were made manually and calculations by computer (Univac 11-08 EXC 8) The mean value and standard deviation of individual results for each parameter were based on five consecutive trackings at each velocity and in each direction Altogether 1 200 trackings were recorded in 20 normal subjects 13 trackings were excluded for technical reasons

RESULTS

The mean value and dispersion of each parameter during voluntary tracking eye movements to the right in 20 normal subjects are given in Table I

The maximum velocity gain of smooth pursuit eye movement (MVSP gain)

The eyes followed the target with exact velocity (MVSP gain = 1 00) in only 7 6% of all trackings

The mean MVSP gain was close to *1 00* at target velocities of 10–40 s^{-1} and occasionally at target velocities of 50 s^{-1} and 60° s^{-1} but as a rule the MVSP gain decreased with increasing *target velocity* (Fig 2) Slight MVSP gain overshoots i e gains of 1 01–1.30, were found in 22% of the trackings at both low and at high target velocities In most of the trackings (70 4%) however the eyes lagged behind the visual target The interindividual variation increased with increasing stimulus velocity The variation coefficient was 15% at target velocities of 10–40° s^{-1} As the target accelerated the variation *coefficient* could exceed 30% The intra individual range

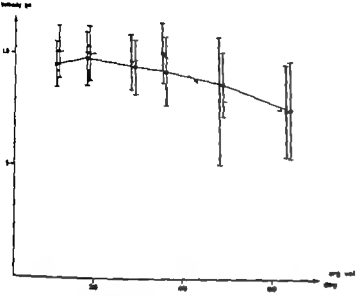


Fig 2 Maximum velocity gain of smooth pursuit at different velocities of the visual target. Means of individual means ± S D in 20 normal subjects during tracking to the right (—) and to the left (- -)

of variation was also velocity dependent up to a target velocity of 40° s the variation coefficient between gains in the separate trackings was less than 10% in the majority of subjects At a target velocity of $40° s^{-1}$ or higher the variation of MVSP gain increased abruptly in most subjects and often exceeded 30%

No statistically significant difference in MVSP gain between tracking to the right and to the left could be found (*t*-test for unpaired data, $p>0.05$)

Amplitude of smooth pursuit eye movements (ASP)

The total tracking amplitude (TA) gradually decreased in inverse proportion to the increasing velocity of the visual target corresponding to the decrease in amplitude of the returning saccade according to the construction of the test Parallel with the increase in target velocity there was a percentage decrease in the amplitude of smooth pursuit This decrease was of the same range at target velocities of

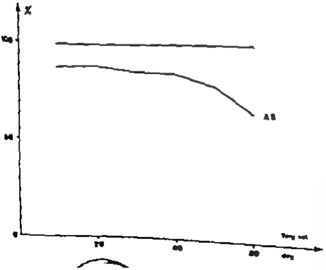


Fig 3 Amplitude of smooth pursuit (*ASP*) as percentage of total tracking amplitude (*TA*) at increasing velocities of the visual target during voluntary tracking eye movements.

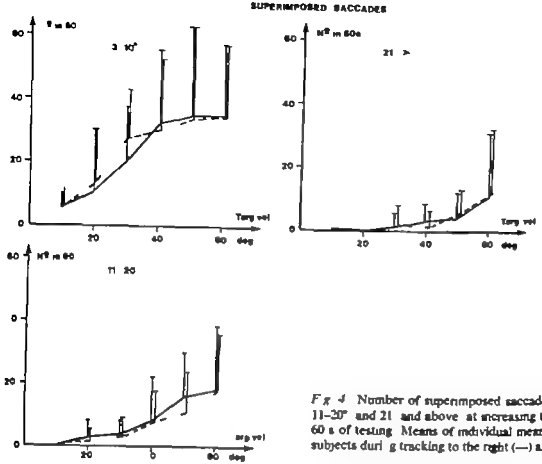


Fig 4 Number of superimposed saccades of amplitudes 3-10° 11-20° and 21 and above at increasing target velocities, during 60 s of testing Means of individual means ± S D in 70 normal subjects duri g tracking to the right (—) and to the left ( )

10–40° s⁻ while above the target velocity 40° s^{-1} the percentage decrease in smooth pursuit was more abrupt (Fig 3) to favour the sac cadic mode of tracking No significant direc tional difference in ASP could be found (*t* test for unpaired data $p>0$ 05)

Superimposed saccades (SS)

Superimposed saccades were present in most subjects—on the average 0 9/tracking In 3 of the subjects however the saccade free track ings were recorded The majority of saccades 80% belonged to the smallest amplitude cathegory 3–10° 15% of SS were in the range 11–20° and only 5% were larger than 20° The frequency and amplitude of SS were related to the velocity of the target saccades of 3–10° increased appreciably with increasing target velocity Saccades of 11–20° became more common at target velocities of 30° s and above and finally saccades larger than 20° appeared only at velocities of 30° s^{-1} and above (Fig 4) There was no statistically significant difference in frequency of SS to the right or to the left (*t* test for unpaired data $p>$ 0 05)

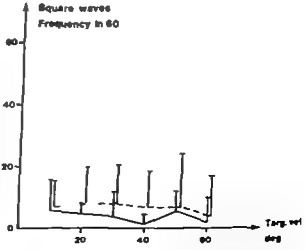


Fig 5 Frequency of square w es at different target elocities during 60 of testing Mean of individual means + S D in 70 normal subject during tracking to the right (—) and to the left ( )

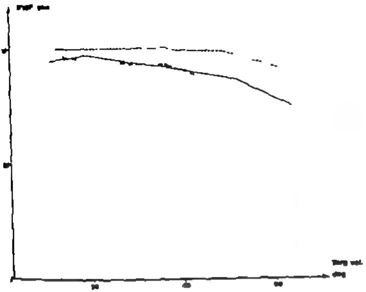


Fig 6 Effects of repeated testing on maximum elocity gain of smooth pursuit (MVSP gain) Means of individual means in 9 subjects investigated twice were higher on the repeated testing ( ) compared to the first occasion ( ). The reference group (—) of 20 normal subjects was in estigated only once

Additional testing group

Nine additional subjects were tested twice at intervals of 1–7 days On the first occasion there was no statistically significant difference in the mean results compared with the main group of 20 subjects with respect to MVSP gain ASP SS and SW However on the second occasion MVSP gain and ASP (Figs 6 and 7) were significantly higher (*t*-test for paired samples $p<0.05$) at all target velocities except 60° s . Also there were more SS of 3–20° during the second test while the frequency of SS with amplitudes of 21 and above remained constant (Fig 8). There was no statistically significant difference in the frequency of SW during the first vs the second investigation

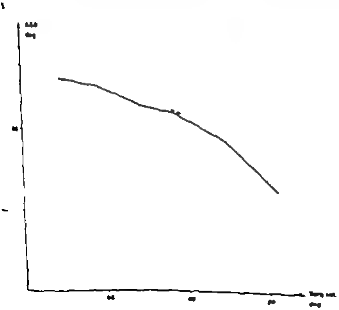
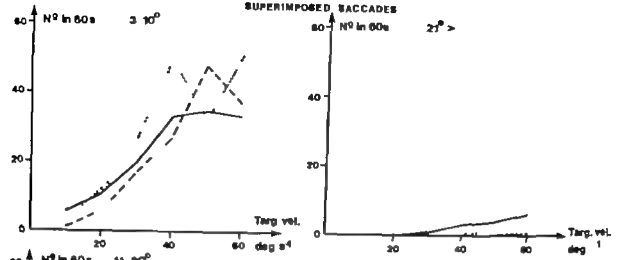


Fig 7 Effects of repeated testing on amplitude of smooth pursuit. Means of individual means in 9 subject investigated on the first ( ) compared ith the second ( ) occasion, and to the reference group of 20 normal subject (—) investigated once.



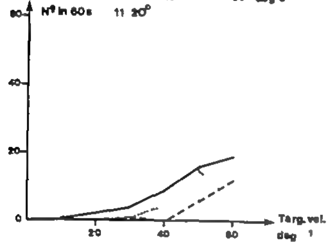


Fig. 8. Effects of repeated testing on frequency of superimposed saccades. Means of individual means in 9 normal subjects investigated on the first occasion ( ) compared with results of the second testing ( ) and to a reference group of 70 normal subjects (---).

DISCUSSION

Adaptation of eye velocity to velocity of the target during smooth pursuit

Eye velocity, even during a single tracking, seldom remains constant and shows considerable variation (Westheimer 1954, Brown 1972, Williams & Fender 1979). Different arbitrary parameters have therefore been used to express eye velocity during smooth pursuit, e.g. mode eye velocity (Baloh et al. 1976) and average velocity (Brown 1972, Kowler et al. 1978). In the present paper the maximum velocity of smooth pursuit was chosen in order to limit sources of error due to possible temporary slackening of attention. The mean values of maximum velocity gain of smooth pursuit (MVSP gain) at different target velocities were comparable to results obtained by other authors (Baloh et al. 1976, Sharpe et al. 1979). In agreement with the earlier investigations the mean MVSP gain decreased in inverse proportion to increasing target velocities.

The present material indicates the limited ability of the eyes to match velocity of the target: the ideal gain was achieved in only 7.6% of the trackings, in 70.4% eye velocity was lower than that of the target and velocity overshoots arose in 22% of the trackings. Williams & Fender (1979) proposed recently that the poor velocity adaptation and velocity overshoots derive from the sloppy character of the smooth pursuit control system. It has also been shown that both high velocity gains and overshoots arise mostly when tracking targets move in a predictable manner (Stark et al. 1962, Kowler et al. 1978, Sharpe & Sylvester 1978). As the present test used predictive tracking, the velocity overshoots might be, at least partly, a result of overcorrection of anticipated velocity error.

Visual perception of target during tracking eye movements

As each subject can deliberately start or interrupt tracking eye movements at any moment instantaneously, it was considered of interest to monitor the overall excursion of the eyes during tracking (TA) as well as the amplitude of smooth pursuit (ASP). Since perception of the moving object is possible only during pursuit (Yarbus, 1967) the ASP is expected to reflect the extent to which the target was seen during the tracking. Hence, the target can be visually monitored to roughly the same degree at target velocities ranging from 10° to 40°/s (during 85–89% of each tracking), whereas continuous visual control of the moving target falls off abruptly at higher target velocities, the decrease of visual perception, the velocity gain was seldom close to 1.00 and an abrupt increase in inter- and intra-individual variations was observed (Fig. 2). At the same time saccades covered more than 20% of total tracking amplitude (Fig. 3). Tracking entirely free from saccades could also occur, as observed in 3 of the subjects, indicating that the smooth pursuit system can function above optimal velocity limits. This type of tracking may be explained by a high degree of attention and motivation (Kommerell & Täumer, 1972; Troost et al., 1972). In most of the subjects, however, an increase in saccadic frequency was observed with increasing target velocities, thus supporting the theory that the saccadic system has to be activated when the smooth pursuit system itself cannot adequately control the position of the eyes (Williams & Fender, 1979).

The saccades produced in order to catch up with the target are believed to be induced either by retinal information on change in target position (Rashbass, 1961) or else extraretinally, due to anticipated positional error of the eyes (Zee et al., 1974).

In the present material most of the saccades seemed to be induced and controlled by retinal mechanisms as saccadic amplitudes were related to the velocity of the target. Moreover, the frequency of saccades allowed time courses sufficient for the retinal signal processing (see Monnier, 1952). However, occasionally saccades could be very frequent, appearing at intervals shorter than the minimal 150 ms (Stark et al., 1962), especially at target velocities of 50° and 60°/s. Hence, the anticipatory mechanism of saccadic control must be considered. It is proposed that both the retinal and the extraretinal mechanism of control of superimposed saccades are functioning during voluntary tracking. The anticipatory mechanisms seem to be activated most ly at high velocities of the visual target.

Characteristics and origin of square waves

In contrast to the observed increase in superimposed saccades, the number of square waves remained fairly constant at different target velocities (cf. Figs. 4 and 5). Another difference between SS and SW was that saccades were always directed with the target, whereas primary saccades in the SW could occasionally turn the eyes in the opposite direction. These observations suggest that saccadic components in SW are not produced in order to correct eye velocity or any other constant error of smooth pursuit. The eyes would appear to jump randomly during the SW, possibly reflecting a random activity of pontine neurons in normal subjects.

Co-operation of smooth pursuit and saccadic systems in maintaining eye and target position

When one's interest focuses on a moving target the eye motor system receives the information of target position and velocity. Both the smooth pursuit and the saccadic subsystems of eye movement control are activated. Continuous visual feed-back is necessary to fix the eyes on the target during smooth pursuit (Robinson, 1965). The present results indicate a velocity of 40°/s as the limit for optimal smooth pursuit. Above this range, along with

Effects of repeating the test

A significant change in the mean results of the 9 subjects investigated twice was found on

repeated testing i e higher MVSP gains and ASP as well as a higher frequency of saccades of 3–20° This could be interpreted as an effect of training in motivated subjects (de Weese Puckett & Steinman 1969) The change in eye movement function seems to apply to both velocity matching and correction of positional errors

CONCLUSIONS

In the present work an effort to quantify voluntary tracking has been made by applying four parameters maximum velocity gain of smooth pursuit (MVSP gain) amplitude of smooth pursuit (ASP) frequency of superimposed saccades (SS) of different amplitude and frequency of square waves (SW)

An adequate following viz an MVSP gain close to 1 00 an ASP close to overall tracking amplitude and a limited number of SS and SWs were recorded mainly at low target velocities A target velocity of 40° s^{-1} appears to be the limit for optimal functioning of the smooth pursuit system At higher velocities the visual control of the target s movement decreases and the saccadic system will be activated to an increasing extent Superimposed saccades at target velocities above 40° s^{-1} seem to be triggered to some extent due to extraretinal control of targets position

Square waves appear to occur randomly and without any corrective function during the tracking eye movements

Training activates the smooth pursuit system as well as the saccadic system to perform the voluntary tracking with desired precision

A forthcoming study will elucidate the extent to which the different parameters of tracking eye movements are affected in patients suffering from labyrinthine and central nervous system disorders

ZUSAMMENFASSUNG

Vom Willen abhängige und verfolgende Augenbewegungen wurden bei 20 normalen Personen analysiert welche mit dem Blick einem beweglichen optischen Stimulus bei sechs verschiedenen konstanten Geschwindigkeiten zwischen 10 und 60° s folgten. Die Fähigkeit zur verfolgenden Augenbewegung wurde mit Hilfe von vier Parametern quantifiziert. Der Durchschnittswert und die Ausbreitung jedes Parameters wurde bei jeder Geschwindigkeit bestimmt. „Gain der maximalen Geschwindigkeit von „smooth pursuit" betrug durchschnittlich 0.98–0 75 und verminderte sich stufenweise mit zunehmender Geschwindigkeit des optischen Gegenstandes *Die Amplitude von „smooth pursuit"* reduzierte sich ebenfalls sukzessiv wenn die Geschwindigkeit des Gegenstandes zunahm und „smooth pursuit" durch immer frequentere Saccaden ersetzt wurde Saccaden mit Amplituden von 3–10° kamen bei allen Geschwindigkeiten vor während Saccaden großer als 10° oft bei Geschwindigkeiten über 30° s auftraten Square waves waren selten, erschienen aber mit der gleichen Frequenz, unabhängig von der Geschwindigkeit des Gegenstandes und sie waren anscheinend zufällig ausgelöst worden

Eine zweite Gruppe von neun Personen wurde bei zwei separaten Gelegenheiten untersucht. Die Durchschnittswerte des „gain der maximalen Geschwindigkeit von smooth pursuit" die Amplitude von „smooth pursuit" sowie die Frequenz von Saccaden waren höher bei der zweiten Untersuchung wahrscheinlich als Wirkung des Einlernens

Die Kooperation zwischen den beiden Systemen der Augenbewegung – saccadische und „smooth pursuit" – während der vom Willen abhängigen verfolgenden Augenbewegungen wurde diskutiert.

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Lennart Schalén, M.D.
Department of Otorhinolaryngology
University Hospital of Lund
S-221 85 Lund
Sweden

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COMPENSATION DES DÉFICITS POSTURAUX ET CINÉTIQUES APRÈS NEURECTOMIE VESTIBULAIRE UNILATÉRALE CHEZ LE CHAT

Role de l'activité sensorimotrice

C Xerri et M Lacour

Laboratoire de Psychophysiologie Université de Provence Centre St Jérôme Marseille France

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Résumé Le rôle de l activité sensori-motrice dans la compensation des déficits labyrinthiques a été étudié par une méthode comportementale chez 4 chats adultes Les déficits de la posture et de l équilibre cinétique sont quantifiés et leur compensation ultérieure est décrite Le décours de la récupération obtenu chez des animaux laissés libres de leur activité exploratoire après neurectomie vestibulaire unilatérale a été comparé à celui d'animaux soumis à une période de restriction sensorimotrice (R. S M) d'une durée de 7 jours située à différents moments post-opératoires Chez l'animal libre les asymétries posturales se compensent en trois périodes distinctes aboutissant vers 40 jours à une symétrie de type pré opératoire La récupération de l'équilibre cinétique évolue par paliers et s achève en 50 jours. Seule une R. S M précoce (2ème au 9ème jour) suspend la récupération de la symétrie posturale La R S M suspend la récupération de l'équilibre cinétique et induit un retard d'autant plus prononcé qu elle est plus précoce Une période de sensibilité du Système Nerveux aux d verses sources de vicariance est suggérée

Abstract The role of sensorimotor activity in compensating deficits following unilateral vestibular neurectomy was studied in four adult cats sing behavioral tests Disturbances in posture and equilibrium were quantified and their subsequent compensation was described in both sensorimotor restrained and unrestrained cats Sensorimotor restriction (S M R) lasted 7 days and was performed in different postoperative periods In the unrestrained animal postural asymmetry compensation followed a 3-phase t me course leading to preoperative criteria after about 40 days Recovery of equil bri m developed by steps and was achieved after about 50 post operative days A first week applied S.M R was most effective in stopping postural ymmetry recovery while a later S M R. had no effect on the recovery time course and did not produce decompensation On the contrary S M.R (1st week or 3rd week) prevented and delayed equilibrium recovery the earlier S M R produci g ma i-mal effects These observations suggest a CNS sensitive period" to vicariant inputs

L exclusion unilatérale des afférences labyrinthiques engendre des déficits posturaux locomoteurs oculomoteurs (Flourens 1842 Magnus 1922 1924 Rademaker 1935 Thomas 1940) et réflexologiques (Lacour et al 1976 1978) qui se compensent ultérieurement De nombreux travaux ont mis en évidence l intervention du cervelet (McCabe & Ryu 1969) du cortex cérébral (Kolb 1955), de l olive inférieure (Llinas 1975) des noyaux vestibulaires (Precht et al 1966 Precht 1974) de la vision (Ewald 1892 Magnus 1924 Dow 1938 Courjon et al 1977 Putkonen et al 1977) du labyrinthe intact (Lacour et al 1979) dans la compensation des déficits labyrinthiques Un rôle important dans la compensation semble également dévolu à la moelle épinière et aux afférences somatiques Une section des racines dorsales ou de la moelle épinière au niveau thoracique retarde la compensation des déficits labyrinthiques (Kolb 1955 Schaefer & Meyer 1974) ou provoque une brève réapparition des symptômes labyrinthiques chez l'animal qui a totalement compensé (Azzena 1969 Azzena et al 1976 1977) Un effet de retard est observé chez le singe immobilisé immédiatement après hémilabyrinthectomie pendant une période de quatre jours (Lacour et al 1976)

Nous avons étudié chez le chat le rôle des afférences somatiques suscitées par l'activité motrice dans le développement de la récupéra-

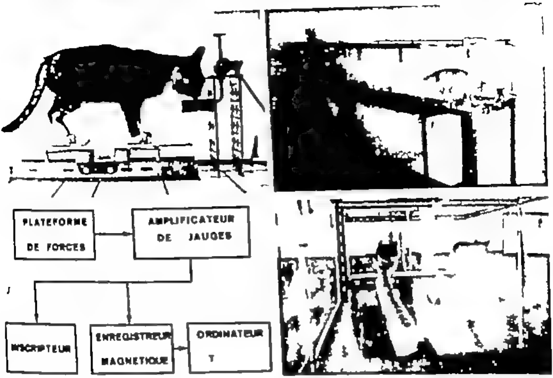


Fig 1 Dispositifs expérimentaux. Plate-forme de forces et diagramme du dispositif d'enregistrement et de traitement des données (à *gauche*). Dispositif de la poutre tournante (à *droite*).

tion fonctionnelle après neurectomie vestibulaire unilatérale. Des tests comportementaux ont été utilisés pour quantifier les asymétries posturales et les déficits de l'équilibre cinétique. Le décours de la récupération obtenu chez des animaux laissés libres de leur activité exploratoire après opération a été comparé à celui d'animaux soumis à une période de restriction sensorimotrice (R.S.M.) située à différents moments postopératoires.

MATÉRIEL

Une plate-forme de forces et une poutre tournante ont été utilisées pour quantifier respectivement les asymétries posturales et les déficits de l'équilibre cinétique.

Plate-forme de forces (Fig. 1 gauche)

La plate forme est composée d'une feuille d'aluminium rectangulaire (35×20 cm) qui repose sur quatre pointeaux solidaires d'un axe en cuivre au béryllium. Des repères disposés sur la plate-forme définissent une position standard des pattes de l'animal. Chacun des axes est équipé de deux jauges de contrainte (type FLA 600-11 120±0,3 Ω). Les quatre axes sont fixés sur un support central qui stabilise l'ensemble du dispositif.

Poutre tournante (Fig. 1 droite)

Deux compartiments sont reliés par une poutre horizontale située dans un tunnel. Les compartiments (50×60×50 cm) et le tunnel sont en plexiglas transparent. L'ensemble, long de 3 m, est placé à 1,20 m au-dessus du sol. La poutre (longueur 2 m, diamètre 12 cm) est entraînée par un moteur électrique et peut tourner sur son axe central avec une vitesse linéaire variable (0 à 25 m/mn). Une lampe fixée au plafond de chacun des compartiments est utilisée pour conditionner les animaux à traverser la poutre. Chaque compartiment

contient une écuelle distribuant du lait par commande d une électrovanne

Système de contention

Une cage métallique (30×15×20 cm) est utilisée pour limiter les activités sensori-motrices de l animal Le chat est privé d activités locomotrices et ne peut se tenir debout sur ses pattes Il peut seulement se retourner dans la cage en position couchée L animal dispose normalement des informations visuelles Ce système de contention assure une restriction sensori motrice tolérable qui n entraîne pas d altérations du comportement

MÉTHODES

Procédure pré-opératoire

Plate forme Après une courte période d apprentissage (5 à 6 séances de 5 min) le chat placé sur la plate-forme adopte spontanément une attitude érigée et symétrique et la maintient tant que dure le renforcement par distribution de lait (période de 10 sec) On prend une mesure instantanée de la répartition des forces d appui et on enregistre leurs variations pendant 10 sec On répète cette procédure six fois

Poutre Le chat est conditionné à traverser sur la poutre lorsque la lampe de son compartiment s allume Il reçoit du lait une fois arrivé dans le compartiment opposé (renforcement positif) Les premiers passages s effectuent poutre immobile puis on incrémente la vitesse linéaire de la poutre de 1 m/min après quatre passages consécutifs sans chute On mesure au chronomètre le temps de passage pour chaque vitesse de rotation

Les performances évoluent au cours des essais la vitesse limite de la poutre s accroît régulièrement et la vitesse de passage du chat se réduit progressivement Ces performances se stabilisent en fin d apprentissage (entraînement quotidien d'une demi-heure à une heure durant quatre semaines) A la fin de l apprentissage trois chats ont été opérés (neurectomie vestibulaire du côté droit) le quatrième ayant servi de témoin

Neurectomie vestibulaire

La neurectomie vestibulaire s effectue au microscope opératoire sous anesthésie générale (injection intrapéritonéale de 40 mg/kg de pentothal) La technique utilisée est celle décrite par Curthoys et al (1971) On accède à l oreille interne par la bulla tympanique puis on expose le nerf auditif et les nerfs vestibulaires inférieur et supérieur La neurectomie vestibulaire est alors effectuée et le conduit obturé

Procédure post opératoire

Les animaux ont été testés tous les deux ou trois jours durant une période de deux mois environ Pendant les premiers jours post opératoires le chat ne peut traverser la poutre même immobile On utilise alors un pont à largeur variable (40 30 20 et 10 cm)

Les trois chats opérés ont été soumis à une restriction sensori motrice (R S M) de 7 jours Un chat a été immobilisé du 2ème au 9ème jour (R S M 1) un second du 16ème au 23ème jour (R S M 2) et un troisième du 50ème au 57ème jour (R.S M 3) lorsque sa récupération a été jugée complète à la plate forme et à la poutre Le chat témoin non opéré a été soumis en fin d'apprentissage à une même R S M de 7 jours Son entraînement a également été interrompu pendant une autre période de même durée afin de déterminer sur le chat intact les effets possibles de la R S M et de dissocier chez l animal opéré les effets de la R S M de ceux attribuables à l'interruption de l entraînement

Traitement des données

Le degré de symétrie posturale est établi en exprimant en pourcentage le rapport des forces exercées au niveau des trains antérieurs et postérieurs (avant gauche/avant droite ×100 et arrière gauche/arrière droite ×100) On calcule un rapport moyen pour chaque train par session expérimentale Des limites de confiance sont calculées à 0 05 Un ordinateur T 1600 fournit pour chaque session des histogrammes de position en X (déplacement la

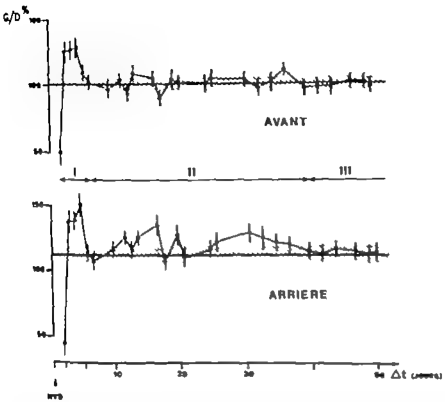


Fig. 2 Évolution post-opératoire de l'indice de symétrie posturale en l'absence de restriction sensori-motrice. L'indice du degré de symétrie posturale (rapport des forces exercées par les pattes gauche et droite G/D 100) est exprimé (6 valeurs par point) pour les trains antérieur et postérieur en fonction du temps post-opératoire (Δt en jours) à partir de la date de la neurectomie vestibulaire (V. N. D.). Les tracés horizontaux représentent l'indice moyen de symétrie (=24 valeurs) obtenu avant opération. Des limites de confiance sont calculées à 0,05 (bande hachurée pour les tracés horizontaux, lignes verticales pour les points de chaque courbe). Les 3 phases caractéristiques de l'évolution de l'indice de symétrie sont indiquées (*I*, *II* et *III*)

téral) des stabilogrammes X–Y et des histogrammes de vitesse de variation des forces en X établis sur une période d'analyse de 10 sec.

A la poutre tournante on détermine la performance maximale (P_{max}) de chaque animal par session expérimentale : elle correspond à la vitesse maximale de la poutre qui n'entraîne pas de chute pour quatre passages consécutifs de l'animal. On exprime la P_{max} de chaque session post-opératoire en pourcentage de la P_{max} moyenne réalisée au cours des quatre dernières sessions pré-opératoires. On calcule à chaque délai post-opératoire la vitesse moyenne du chat (V) pour chacune des vitesses de rotation de la poutre (V). Ces résultats sont comparés aux données des quatre dernières sessions pré-opératoires.

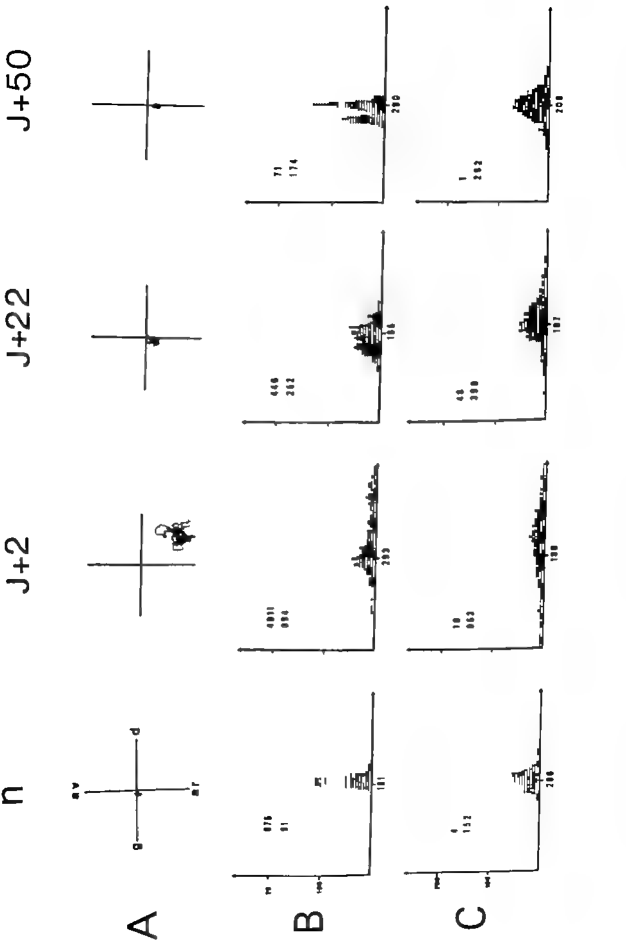
RÉSULTATS

1. *Compensation des déficits posturaux*

a. En absence de R. S. M. (chat libre après opération)

Le degré de symétrie posturale observé chez le chat non soumis à R. S. M. évolue en trois périodes distinctes dans les trains antérieur et postérieur (Fig.)

Période I (? à 7 jours) : la posture est caractérisée par de profondes asymétries. Du 2ème au 3ème jour l'animal déporte son poids essentiellement sur les pattes droites ipsilatérales à la section. Les forces exercées par les pattes gauches ne représentent qu'environ 50% des valeurs contrôles. Un tel degré d'asymétrie provoque des chutes fréquentes vers le


A
B
C
n
J+2
J+22
J+50

côté droit. Du 3ème au 7ème jour l'asymétrie est toujours importante mais elle s'inverse l'animal déporte son poids sur ses pattes gauches (130–140 % des valeurs contrôles)

Période II (7 à 40 jours) on observe de 7 à 1? jours un retour rapide mais peu durable à un degré de symétrie posturale de type pré-opéra toire Par la suite on note à nouveau des asy métries significatives mais cependant moins prononcées que celles de la période I Ces asymétries s atténuent progressivement et évoluent selon un décours oscillatoire

Période III (à partir de 40 jours) on note un retour définitif à un degré de symétrie pos turale de type pré-opératoire Le déficit rési duel consiste en une inclinaison de la tête

Les stabilogrammes (Fig. 3 A) et les histo grammes de position en X (Fig. 3 B) illustrent les phénomènes précédemment décrits Au 2ème jour (J+2) le chat déporte son poids en arrière et du côté de la lésion Les déplace ments du centre de gravité sont très im portants Durant la période II (J+22) l'asy métrie posturale se réduit et l'animal se dé porte légèrement vers la gauche Durant la pé riode III (J+50) le chat est à nouveau stable et symétrique Les histogrammes de vitesse en X (Fig 3 C) indiquent que les vitesses de dé placement du centre de gravité sont en moyenne plus lentes et situées dans une gamme plus étendue après opération Elles deviennent progressivement comparables aux valeurs contrôles

Fig 3 Modifications de la symétrie et de la stabilité posturale au cours des 3 périodes post-opératoires carac téristiques chez le chat non-entraîné (A) Les stabilo grammes en X Y montrent que la posture symétrique chez le chat normal ( ) est caractérisée au cours de la première période post-opératoire (*J* 2) par une asymétrie très marquée qui se réduit ultérieurement (*J* 22) La posture est de type pré-opératoire au cours de la 3ème période (*J* 50) On note également après opération une amélioration progressive de la stabilité posturale (B) Les histogrammes de position en X (durée de l'analyse 10) montrent l'évolution des phénomènes décrits en (A) *En abscisses* nombre de classes (classe modale indiquée) *En ordonnée* effectif par classe La médiane (m) et écart semi-interquartile) sont indiqués en m\ sur chaque histogramme (C) Les histogrammes de vitesse en X durée de l'analyse 10 montrent que les vitesses de déplacement du centre de gravité de l'animal sont en moyenne plus lentes et situées dans une gamme plus étendue après opération Elles deviennent progressive ment comparables aux valeurs contrôles (mêmes conven tions que pour (B))

b *Effets des R S M appliquées à différents moments post-opératoires*

L évolution du degré de symétrie posturale chez le chat soumis à une R. S M durant la première semaine post-opératoire (Fig 4 A) présente les caractéristiques suivantes

Période I (9 à 12 jours) l'asymétrie pos turale mesurée avant la R. S M (0–2 jours) est identique à celle observée à la même période chez l animal libre (cf Fig 2) Au 9ème jour on observe toujours un appui important sur les pattes droites La R. S M a suspendu la ré cupération posturale. L asymétrie s atténue au cours des 3 jours suivants On note l'absence de l inversion de l asymétrie observée chez le chat libre

Période II (12 à 37 jours) on observe une brève période de symétrisation suivie d asy métries peu marquées présentant un décours temporel oscillatoire plus prononcé dans le train postérieur

Période III (à partir de 37 jours) on assiste au retour définitif à un degré de symétrie de type pré-opératoire

La R. S M appliquée durant la 3ème se maine post-opératoire (Fig. 4 B) n a pas mo difié le décours de la récupération. La R. S M effectuée sur le chat libre après complète ré cupération (Fig 4 C) n a aucun effet on n observe pas de décompensation Nous n avons pas non plus observé d'effet d'une R. S M de 7 jours ou d'une interruption de l entraînement de même durée chez le chat in tact.

Compensation des déficits de l'équilibre

a. *En absence de R S M* (chat libre)

La performance maximale (P_{max}) est très al térée après opération Chez l'animal libre la récupération s achève par paliers en 50 jours jusqu au rétablissement de la P_{max} pré-opéra toire (Fig. 5 A) L animal est incapable de tra verser la poutre immobile avant 1? jours. Du

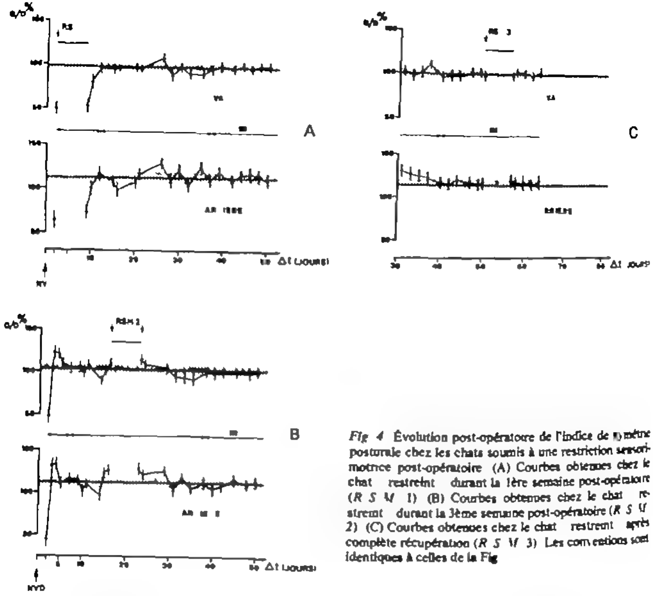


Fig 4 Évolution post-opératoire de l'indice de symétrie posturale chez les chats soumis à une restriction sensori-motrice post-opératoire (A) Courbes obtenues chez le chat restreint durant la 1ère semaine post-opératoire (*R S M* 1) (B) Courbes obtenues chez le chat restreint durant la 3ème semaine post-opératoire (*R S M* 2) (C) Courbes obtenues chez le chat restreint après complète récupération (*R S M* 3) Les conventions sont identiques à celles de la Fig

4ème au 5ème jour il ne peut traverser le pont que pour une largeur de 40 cm progressivement il peut le traverser pour une largeur de 10 cm (10 jours) Pendant les 20 premiers jours post-opératoires la récupération de la P_{max} demeure partielle (30%) mais rapide Elle se stabilise ensuite jusqu à la fin du premier mois et s établit rapidement du 31ème au 49ème jour ou elle devient complète

b *Effets des R S M appliquées à differents moments post-opératoires*

La récupération diffère selon le moment post opératoire d application de la R S M La restriction précoce (R S M 1 Fig 5C) suspend la récupération qui ne se développe que lors que cesse l immobilisation (9ème jour) Elle s établit comme celle du chat libre jusqu au 14ème jour et par la suite elle est très retardée à 41 jours la compensation reste partielle (15%) et à 50 jours l'animal n a récupéré que 40% de sa P_{max} pré-opératoire

La R S M 2 (Fig 5B) a bloqué la récupération à son niveau initial (fin de la 2ème semaine) Lorsque cesse l immobilisation elle s établit de nouveau mais plus lentement que chez le chat resté libre Le retard est net mais cependant moins marqué que celui observé après R S M 1 A 50 jours l animal est à 50% de sa P_{max} pré-opératoire

Lorsque la restriction est effectuée après complète récupération de la P_{m} (R S M 3 Fig 5A) elle n entraîne pas de modifications. De même la stratégie adoptée par l'animal

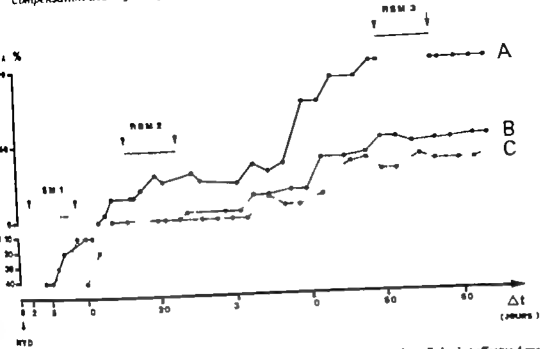


Fig 5. Évolution de la performance maximale (P_{max}) à la poutre tournante : effets d'une restriction sensori-motrice de 7 jours effectuée à différents moments post-opératoires. *En abscisses* : Temps post-opératoire (Δt) exprimé en jours à partir de la date de la neurectomie vestibulaire (N V D). *En ordonnée* : P_{max} post-opératoire exprimée en % de la P_{max} pré-opératoire obtenue en fin d'apprentissage. La P_{max} est définie comme la vitesse maximale de la poutre pour laquelle le chat effectue 4 passages consécutifs sans chute. L'échelle de 40 à 10 cm indique la largeur du pont qui rejoint les deux compartiments (l'animal étant incapable de se déplacer sur la poutre même immobile). Les dates d'application des R S M sont indiquées chez les 3 chats A (*R S M 3*), B (*R S M 2*) et C (*R S M 1*) par des flèches.

reste inchangée : la vitesse de déplacement du chat n'est pas modifiée. Par ailleurs, ni une R.S.M., ni un arrêt de l'entraînement de même durée ne modifient les performances chez le chat témoin.

c Apprentissage – Réapprentissage

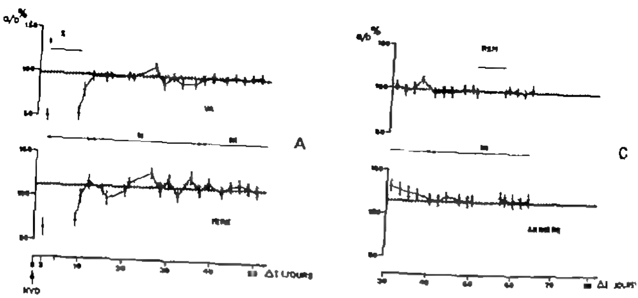
La figure 6 décrit l'évolution pré- et post-opératoire des courbes de vitesse de déplacement des chats en fonction de la vitesse de rotation de la poutre. Durant la période pré-opératoire les chats traversent d'abord très rapidement la poutre. Progressivement ils réduisent leur vitesse de déplacement et améliorent leur P_{max}. Ces performances se stabilisent en fin d'apprentissage (4ème semaine). Après opération le chat libre traverse d'abord rapidement la poutre contrairement aux chats soumis à une R. S. M. Ultérieurement on note de nouveau chez deux animaux une réduction progressive de la vitesse de passage et une augmentation de la P_{max} jusqu'aux valeurs contrôles. Peu de modifications sont observées chez le troisième animal.

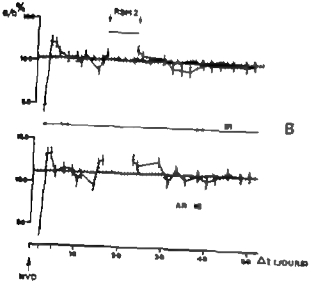
DISCUSSION

L'activité sensori-motrice de l'animal libre semble être un facteur déterminant de compensation. Il semblerait cependant que les afférences somatiques ne jouent pas le même rôle dans l'élaboration et dans le maintien de la compensation.

Rôle de l'activité sensori-motrice dans l'élaboration de la compensation

Lorsqu'on limite les interactions de l'animal avec son milieu durant la première semaine post-opératoire, la récupération est suspendue à son niveau initial et ne commence à se





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Rôle de l'activité sensori motrice dans le maintien de la compensation

La R S M n a pas d'effets lorsqu elle est pratiquée sur un animal qui a récupéré totalement elle ne provoque pas de décompensation Il semble donc que l'activité sensori motrice du sujet et les afférences somatiques qu elle suscite n aient plus, après complète récupération la même valeur adaptative et fonctionnelle Si les réafférences somatiques paraissent nécessaires à l'édition des programmes nouveaux ou à la restructuration des programmes existants elles ne sont plus seules nécessaires à l'entretien de ces programme et par là même au maintien de la compensation Ce point est en partiel désaccord avec les travaux de Azzena (1969) qui montrent une décompensation mais peu durable (quelques heures) lorsqu on sectionne les racines dorsales de la moelle chez le cobaye totalement compensé Une telle décompensation a été également observée par Putkonen et al (1977) chez le chat maintenu dans l'obscurité après une complète récupération en vision normale

Suppléances sensorielles et notion de période sensible

Notre étude suggère qu il est nécessaire pour construire une compensation optimale de disposer très tôt des informations somatiques nées des interactions entre l'animal et son milieu Leur contribution serait essentielle durant les premières semaines post-opératoires à l édition des nouveaux programmes moteurs probablement au même titre que d'autres afférences Elle serait moins importante par la suite les programmes élaborés sur cette base sensorielle plurimodalitaire pouvant alors utiliser d autres sources d'information (afférences visuelles labyrinthiques cutanées) Si l'on considère la compensation des déficits vestibulaires en termes de réapprentissages on peut transposer la conception ancienne de A. Thomas (1940) dans le domaine des *récupérations fonctionnelles* Après hémilabyrinthectomie toutes les sources d'informations disponibles seraient nécessaires à la mise en place d'une riposte rapide du système nerveux La suppression d'une source importante *d'afférences sensorielles* au cours de la période post-opératoire précoce mettrait en échec la construction d un tel dispositif Après réapprentissage une seule source de vicariance (par ex la vision) serait suffisante pour maintenir la *compensation* Cette étude suggère donc ainsi que des résultats obtenus récemment chez le singe (Lacour & Xerri 1980) l'existence d'une période de sensibilité du système nerveux aux diverses sources de vicariance Une analyse neurophysiologique devrait permettre la vérification de ces hypothèses

ACKNOWLEDGEMENTS

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ZUSAMMENFASSUNG

Die Rolle der sensos-motorischen Tätigkeit in der Kompensation de labyrinthischen Defizite ist durch eine Verhaltensmethode bei vier erwachsenen Katzen untersucht worden Die Defizit in der Haltung und im kinetischen

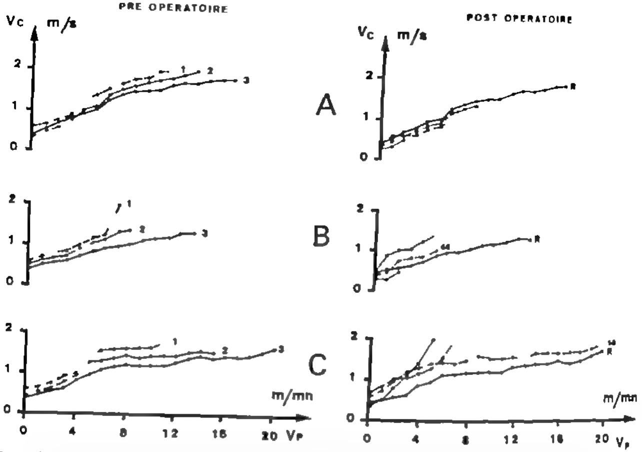

Fig 6 Comparaison de l'évolution des courbes de vitesse de déplacement du chat sur la poutre tournante au cours des périodes pré-opératoire (apprentissage) et post opératoire (réapprentissage). Les séries de courbes représentées pour 3 chats (A B et C) sont obtenues pendant la période pré-opératoire d'apprentissage et pendant la période post-opératoire de réapprentissage *En abscisses* vitesse linéaire de rotation de la poutre (V) en m/mn *En ordonnées* vitesse de déplacement du chat (Vc) en m/s Un intervalle de 15 jours sépare les différentes courbes (*1 2 3*) d'un même animal pendant la période pré-opératoire Après 4 semaines les performances sont stabilisées au niveau 3 Les courbes de vitesse obtenues pendant la période post-opératoire sont représentées à différents moments indiqués en jours par les nombres. La courbe de référence (*R*) est celle obtenue en fin d'apprentissage (courbe 3 période pré-opératoire). Chaque point d'une courbe représente la Vc moyenne obtenue pour 4 essais consécutifs L'évolution post-opératoire observée chez B et C (réduction de la vitesse du déplacement amélioration de la performance maximale) est très comparable à l'évolution des courbes d'apprentissage pré-opératoire

développer qu après libération de l'animal La R S M étant modérée ces résultats suggèrent le rôle important des afférences somatiques nées d une confrontation active avec l environnement dans l élaboration des programmes qui assurent la récupération fonctionnelle Il semble que les possibilités de réorganisation des programmes spécifiques de l équilibration statique soient moins affectées par la limitation précoce des activités sensorimotrices La R S M appliquée au cours de la 3ème semaine n a pas d effet sur le décours de la récupération posturale alors qu elle provoque encore un retard dans la compensation des déficits de l équilibre cinétique Cet effet de retard confirme l'observation clinique princeps de Lacour et al (1976) sur le singe Les afférences somatiques nées du mouvement seraient donc nécessaires à la compensation optimale (rapide et complète) des déficits de l équilibre cinétique

On admet généralement que les asymétries posturales sont dues à un déséquilibre dans la décharge spontanée des noyaux vestibulaires homologues (Precht et al 1966 1974) La restauration rapide d un nouvel équilibre à un niveau de réactivité inférieur et le retour ultérieur à un niveau normal sont également

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Gleichgewicht werden quantifiziert und Ihre spätere Kompensierung beschrieben Die nachoperatorische Entwicklung der Kompensierung bei Tieren die nach einseitiger Neurektomie des 8 Nervs in ihrer Erkundungstätigkeit frei gelassen worden waren ist mit der Entwicklung bei Tieren verglichen worden die einer siebentägigen sensor-motorischen Einschränkung (SME) in verschiedenen Zeitabständen nach der Operation unterworfen worden sind Beim freien Tier beobachtet man drei Perioden in der Kompensierung der Asymmetrien in der Haltung die nach etwa 40 Tagen zu einer Symmetrie von voroperatorischem Typus führen Das kinetische Gleichgewicht stellt sich stufenweise in etwa 50 Tagen wieder ein Nur wenn die SME früh eingeschaltet wird (vom 2 bis zum 9 Tage) verhindert sie die Wiedereinstellung der Symmetrie in der Haltung. Sie hebt die Wiedereinstellung des kinetischen Gleichgewichts auf und die Verspätung die dadurch eintritt ist um so akzentuierter als die SME früher eingeschaltet worden ist Die Vermutung der Existenz einer Periode der Sensibilität des Nervensystems den verschiedenen Vertretungsmöglichkeiten gegenüber wird bekräftigt

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M Lacour
Laboratoire de Psychophys ologie
U ivers te de Provenc
C nt St Jerôme
13397 – Marseill Ced 4
France

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ELEMENTAL COMPOSITION OF THE MATURE INNER EAR

Matti Anniko and Romuald Wroblewski[1]

From the Department of Otorhinolaryngology Karolinska Hospital Stockholm and [1]the Wenner-Gren Institute University of Stockholm Stockholm Sweden

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Abstract An X-ray energy dispersive analysis was performed on the mature serial sectioned freeze-dried mammalian labyrinth (CBA/CBA mouse). The elemental distribution in the endolymphatic and perilymphatic spaces revealed similar results as have been reported in the literature when withdrawing fluid for microanalysis. The cupulae of the vestibular organs showed only minimal relative peak intensities of chlorine and potassium in contrast to the tectorial membrane in the cochlea with elemental identification also of sodium, sulphur and calcium. A difference in structural composition is therefore likely between these two tissues.

The biochemistry of the inner ear has been the object of a great number of investigations during recent years (review Rauch 1964 Thalmann 1976 Silverstein 1976) Considerably less however is known about the relative ion distribution *in vivo* in the various compartments of the labyrinth Microanalysis of inner ear fluids, withdrawn from the fluid-filled spaces in a large number of species all show the specific high potassium content in the endolymph (Smith et al 1954 Citron et al 1956 Silverstein 1966)

Flock (1973 1977) used the technique of electron probe determination concerning the relative ion distribution in the guinea pig cochlea He concluded that ions of endolymph can gain access to the subtectorial space by diffusion through the tectorial membrane which contains the same high potassium content as the endolymph a subject of controversy in the literature (review Ross 1975) A large number of contradictory data are available with regard to electrophysiological measurements (Lawrence et al 1974 review Fex 1974) The fine structure of the tectorial membrane has been analysed in detail by Kronester Frei (1978) finding regional differences with regard to ultrastructural morphology

Whether or not the vestibular hair cells are exposed to endolymph is not known Nor is the elemental composition of the cupula. Differences in ion content between vestibular and cochlear endolymph have been suggested (Silverstein et al 1974)

This study presents an X ray energy-dispersive analysis of the freeze-dried serial sectioned whole inner ear a technique that permits the ions present in the fluid compartments to become entrapped in a network of material thereby reflecting the situation *in vivo* It has to be noted however that the X ray microanalysis of biological material gives only an elemental analysis and the chemical form or activity of the elements—or the compounds that they may represent—has to be based on other independent information. The advantage of this method over histochemical techniques is the simplicity of elemental identification the selectivity and in some cases the ability to quantify the results

MATERIAL AND METHODS

Material

Maturation of the mouse labyrinth occurs within 6–10 days after birth with regard to

This work was supported by grants from Karolinska Institutet, the Swedish Medical Research Council (grant no 17x 730), the Swedish Society of Medical Sciences, and grants from Tysta Skolan

Gleichgewicht werden quantifiziert und ihre spätere Kompensierung beschrieben. Die nachoperatorische Entwicklung der Kompensierung bei Tieren die nach einseitiger Neurektomie des 8 Nervs in ihrer Erkundungstätigkeit frei gelassen worden waren ist mit der Entwicklung bei Tieren verglichen worden die einer siebentägigen sensor-motorischen Einschränkung (SME) in verschiedenen Zeitabständen nach der Operation unterworfen worden sind Beim freien Tier beobachtet man drei Perioden in der Kompensierung der Asymmetrien in der Haltung die nach etwa 40 Tagen zu einer Symmetrie von voroperatorischem Typus führen Das kinetische Gleichgewicht stellt sich stufenweise in etwa 50 Tagen wieder ein. Nur wenn die SME früh eingeschaltet wird (vom bis zum 9 Tage) verhindert sie die Wiedereinstellung der Symmetrie in der Haltung Sie hebt die Wiedereinstellung des kinetischen Gleichgewichts auf und die Verspätung die dadurch eintritt ist um so akzentuierter als die SME früher eingeschaltet worden ist. Die Vermutung der Existenz einer Periode der Sensibilität des Nervensystems den verschiedenen Vertretungsmöglichkeiten gegenüber wird bekräftigt

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M Lacour
Laboratoire de Psychophysiologie
U iversité de Provence
Cent St Jérôme
13397 – Marseille C dex 4
France

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ELEMENTAL COMPOSITION OF THE MATURE INNER EAR

Matti Anniko and Romuald Wroblewski[1]

From the Department of Otorhinolaryngology, Karolinska Hospital, Stockholm and [1]the Wenner-Gren Institute, University of Stockholm, Stockholm, Sweden

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Abstract. An X-ray energy dispersive analysis was performed on the mature serial sectioned freeze-dried mammalian labyrinth (CBA/CBA mouse). The elemental distribution in the endolymphatic and perilymphatic spaces revealed similar results as have been reported in the literature when withdrawing fluid for microanalysis. The cupulae of the vestibular organs showed only minimal relative peak intensities of chlorine and potassium in contrast to the tectorial membrane in the cochlea with elemental identification also of sodium, sulphur and calcium. A difference in structural composition is therefore likely between these two tissues.

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Flock (1973, 1977) used the technique of electron probe determination concerning the relative ion distribution in the guinea pig cochlea. He concluded that ions of endolymph can gain access to the subtectorial space by diffusion through the tectorial membrane which contains the same high potassium content as the endolymph, a subject of controversy in the literature (review: Ross, 1975). A large number of contradictory data are available with regard to electrophysiological measurements (Lawrence et al., 1974; review: Fex, 1974). The fine structure of the tectorial membrane has been analysed in detail by Kronester-Frei (1978) finding regional differences with regard to ultrastructural morphology.

Whether or not the vestibular hair cells are exposed to endolymph is not known. Nor is the elemental composition of the cupula. Differences in ion content between vestibular and cochlear endolymph have been suggested (Silverstein et al., 1974).

This study presents an X-ray energy-dispersive analysis of the freeze-dried, serial sectioned whole inner ear, a technique that permits the ions present in the fluid compartments to become entrapped in a network of material thereby reflecting the situation *in vivo*. It has to be noted, however, that the X-ray microanalysis of biological material gives only an elemental analysis and the chemical form or activity of the elements—or the compounds that they may represent—has to be based on other independent information. The advantage of this method over histochemical techniques is the simplicity of elemental identification, the selectivity and in some cases the ability to quantify the results.

MATERIAL AND METHODS

Material

Maturation of the mouse labyrinth occurs within 6–10 days after birth with regard to

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morphology (Kikuchi & Hilding 1965, 1966) while mature physiology is not attained until the 10th–14th day (Mikaelian & Ruben 1965).

The results of the present study are based on 6 animals sacrificed on the 8th and 14th day respectively post partum.

Methods

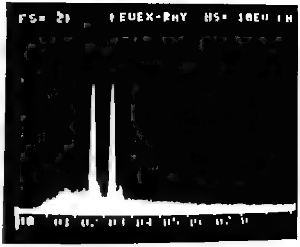
1. *Preparation of sections.* After cervical dislocation the temporal bones were rapidly removed and the bulla was opened. The specimens were thereafter frozen in liquid propane cooled to −190°C by liquid nitrogen. After sectioning of semi-thick sections (16–20 μm) in a cytostat the sections were placed on blocks of pure carbon specimen holders and freeze-dried at −30°C. Conventional histological cryostat sections were alternatingly prepared and stained with haematoxylin-eosin.

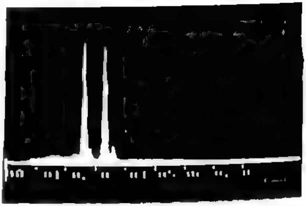
Sections 16–20 μm thick are recommended for this technique to ensure that the X-ray excitation volume is the same throughout the entire section (cf. Wroblewski et al. 1978).

2. *Analytical equipment.* Analytical electron microscopy/electron probe microanalysis was performed with a KEVEX energy dispersive X-ray spectrometer in combination with a JEOL 100C electron microscope provided with a JEOL ASID scanning attachment. The specimens were examined in the scanning mode at 20–40 kV using a take-off angle of 30°. The overall counting time was 50 s. The distance between detector and specimen was 20 mm. During microscopy the cold trap and the cold finger were used. The relative peak intensity (R) for an element was defined as $R_x = (P-b)_x/b$ where $(P-b)_x$ is the number of specific counts measured for element X and b is the background count. For details, see Russ (1974).

RESULTS

Electron probe analysis, presented as emitted energy histograms, was performed either by beam scanning of areas or by counting the number of specific energy quanta at single





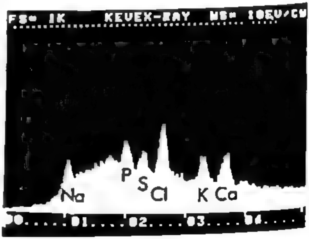
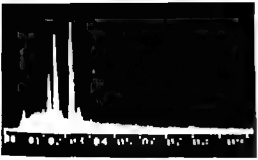
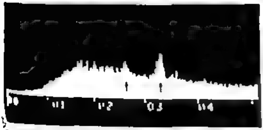


Fig. 1. Emitted energy histogram. Full scale (*FS*) K. Analysis of the content in the endolymphatic space of the cochlear part of the inner ear reveals two distinct peaks, one for chlorine (*left*) and one for potassium (*right*) 8 days after birth (*DAB*).

Fig. 2. Emitted energy histogram. FS 5K, 8 DAB. Endolymphatic space in the vestibular part of the labyrinth. Only chlorine (*left*) and potassium (*right*) can be identified.

Fig. 3. Emitted energy histogram. FS 1K, 8 DAB. Perilymphatic space of the cochlea (scala vestibuli). A large number of elements can be identified as indicated on the histogram.





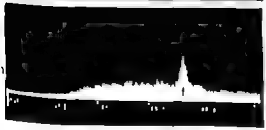


Fig. 4 Emitted energy histogram. FS 2K 14 DAB Tectorial membrane from the basal coil of the cochlea. The analysis is performed near the upper surface of the tectorial membrane The highest peak is identified as potassium followed by chlorine and phosphorus Also sodium (*arrow, left*) and calcium (*arrow, right*) can be identified It has to be noted that no other peaks can be observed in the whole range from approximately 1–9 V

Fig 5 Emitted energy histogram FS 500 14 DAB Cupula of the lateral (*horizontal*) crista ampullaris. Peak can be identified for potassium (*right*) and chlorine (*left*), the former having the highest relative peak intensity N other elements can be identified. The same specimen as in Fig 2

Fig 6 Emitted energy histograms. FS 1K. 14 DAB Measurement of the elemental distribution between the surface of the macula utriculi and the otoconial layer A similar elemental distribution is found as in Fig 5 In the endolymphatic space similar elemental composition was obtained as illustrated in Fig. 2.

Fig 7 Emitted energy histogram. FS 1K. The same specimen as analyzed in Fig. 6. The substance between individual otoconia does not reveal any elements according to this technique. The peak for calcium that distinctly can be observed has been interpreted as scattering effect of emitted energy from calcium when analysing the region close to the otoconia

points studied in the scanning electron microscope Orientation in the specimen was documented both in the electron microscope and by macrophotographs of the whole specimen. Data obtained from the 8- and 14-day-old inner ear respectively were in principle the same

Endolymphatic space

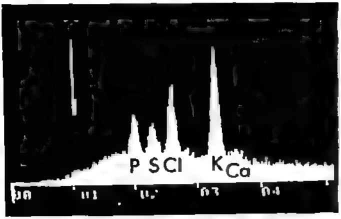
The analysis of endolymphatic material in the cochlea showed two distinct peaks of elemental distribution identified as chlorine and potassium (*K* emission 2 621 and 3 312 eV respectively) A minimal peak is obtained for calcium (*K* emission 3 690 eV) but otherwise no other elements were identified (Fig. 1) Similar results were found in the vestibular part of the inner ear (Fig)

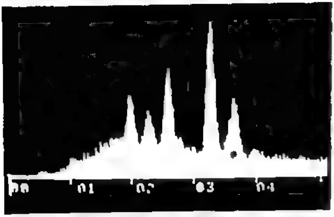
Perilymphatic space

The elemental distribution in this compartment of the labyrinth is no longer as specific as that for the endolymphatic space The most prominent peak is identified as chlorine but also sodium *K* 1 041 eV) sulphur (*K* 2.307 eV) phosphorus (*K* 2.013 eV) potassium and calcium give specific peaks (Fig 3)

The tectorial membrane

This was analysed in detail with regard to regional differences from its insertion in the spiral limbus to its attachment zone at the Hensen cells. The elemental composition was however fairly similar at various regions while still being at the same depth below the endolymphatic space showing very high peaks for potassium and chlorine and also to some extent for phosphorus whereas sodium





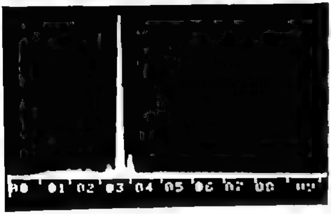


Fig. 8. Emitted energy histogram. FS 1K. 8 DAB. The intracellular elemental distribution is indicated on the histogram. The vertical line above the histogram gives the location for the sodium peak.

Fig. 9. Emitted energy histogram. FS 500. 14 DAB. Apical part of cells of the macula utriculi. Except for the ordinary elemental distribution as expected (see Fig. 8) also a peak is found for calcium (asterisk).

Fig. 10. Emitted energy histogram. FS 5K. 8 DAB. Analysis of otoconia. A distinct peak for calcium is indicated. There occurs no peak for phosphorus.

sulphur and calcium revealed lower but still distinct peaks (Fig. 4). Near the upper surface of the tectorial membrane the relative peak intensity for potassium exceeded the corresponding value adjacent to the underside of the tectorial membrane. The relative peak intensity of sodium was higher near the underside of the tectorial membrane than at its upper surface.

The cupula of vestibular organs

The elemental composition of the cupula differs from that of the tectorial membrane. The cupulae of the cristae ampullares show only minimal peaks for potassium and chlorine while other elements cannot be identified (Fig. 5). A similar elemental distribution is also found in the region between the otoconia and the sensory cell surfaces (Fig. 6). The substance between individual otoconia reveals only a small peak for calcium (Fig. 7) interpreted as being due to scattered energy quanta from otoconial content of calcium.

Hair cell region of the inner ear

Because of the semi-thick sections it was impossible to distinguish in detail hair cells from supporting cells. A typical intracellular milieu was registered in this area with identification of peaks for potassium, chlorine, sulphur and phosphorus (Fig. 8). In the apical part of the hair cell area of the utricle and the saccule in addition a relatively high peak for calcium was observed (Fig. 9). By comparison the otoconia show an extremely high peak for calcium alone (Fig. 10).

DISCUSSION

The elemental distribution in the endo- and perilymphatic spaces according to the present method is in agreement with the results obtained when withdrawing fluid for microanalysis from these two compartments. In comparison the observations of Flock (1977) using the electron probe technique in the guinea pig analysis of perilymphatic material gave only one peak, that for chlorine, while the present study on the CBA/CBA mouse also identifies peaks for sodium, sulphur, phosphorus, potassium and calcium. The present technique does

not, however, allow us to analyse differences in elemental composition between cochlear and vestibular endolymph.

As new findings, the present investigation indicates differences in elemental composition between the tectorial membrane in the cochlea and the cupulae of the vestibular organs. The former has high peaks for chlorine and potassium, a fact that might indicate a penetration of ions from the endolymph through the tectorial membrane to the subtectorial space, thereby exposing the sensory cell surfaces, as also those of supporting cells, to a high extracellular potassium concentration. In addition, distinct peaks for sulphur, phosphorus and calcium were identified which reflect the biochemical composition of the tectorial membrane as has been described by Schätzle (1971), Kleine (1972) and others using histochemical methods.

The X-ray microanalysis studies by Ross (1975) on the upper and lower sides of the rat tectorial membrane indicated a difference in relative ionic concentration. On the upper side potassium and chlorine were present in almost equal amounts. A minimal peak for sodium was also identified. On the underside of the tectorial membrane, chlorine was the most plentiful element, followed by potassium and sodium. Similar results were obtained in our serial sectioned specimens.

In contrast to the tectorial membrane, the cupulae of the vestibular organs have only minimal peaks of potassium and chlorine, being devoid of other identifiable elements. The identification of potassium and chlorine only may reflect the ionic distribution in the small canals of the cupula as morphologically described by Wersall (1956) being in contact with the endolymphatic space (review: Dohlman, 1971; Lim, 1977). Thus the vestibular hair cells too would be exposed to a high extracellular potassium content. The present findings support the observations made by Flock (1977) that the substance of the cupula contained potassium and chlorine in proportions similar to endolymphatic material. According to our study, however, the cupula has a considerably smaller relative peak intensity of these ions than has the tectorial membrane and the surrounding vestibular endolymph. It might even be so that the material of the cupula itself is impermeable to ions, which obtain access to the hair cells via the canals mentioned above. These types of canal are lacking in the tectorial membrane, which therefore might be characterized by another type of ionic permeability, thus giving a similar microenvironment for the physiological function of cochlear and vestibular hair cells. A potassium–chlorine-rich environment above the sensory epithelium has also been recorded from the lateral line organ (*Xenopus laevis*) (Russell & Sellick, 1976).

It has to be noted that there was no difference in elemental distribution between specimens investigated 8 and 14 days, respectively, after birth. This reveals that the specific composition of endolymph is present at the time when morphological maturation of the stria vascularis is completed but before the rapid increase in cochlear and VIII nerve potentials (Kikuchi & Hilding, 1965, 1966; Mikaelian & Ruben, 1965). Similar results were reported by Bosher & Warren (1971) showing that the endolymphatic ionic concentration remained unchanged during the phase of rapid increase in the endocochlear potential (EP). It was indicated that the distinctive endolymphatic ionic composition and the EP arise largely independently and in succession during cochlear maturation. The stria vascularis was morphologically fully mature before the period of rapid change in the EP.

ZUSAMMENFASSUNG

Eine Röntgenenergieanalyse ist auf den voll entwickelten Serienschnitten des vakuum-gefriergetrockneten Säugetierlabyrinths (CBA/CBA Maus) ausgeführt worden. Die elementäre Distribution in den endolymphatischen und perilymphatischen Arealen weist gleichartige Resultate auf wie die in der Literatur berichteten, wenn Flüssigkeit für Mikroanalyse entzogen wurde. Die Cupula der vestibulären Organe weisen nur nominale relative Intensitäten von Chlor und Kalium auf im Gegensatz zur Tectorialmembran in der Cochlea mit elementärer Identi-

fizierung von auch Natrium Schwefel und Kalzium Eine Differenz in der Komposition dieser beiden Gewebe ist deshalb wahrscheinlich

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M tti Anniko M.D
Department of Otorhinolaryngology
Karolinska sjukhuset
S-10401 Stockholm
Sweden

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COMPARISON OF HUMAN SUBJECTIVE AND OCULOMOTOR RESPONSES TO SINUSOIDAL VERTICAL LINEAR ACCELERATION

G Melvill Jones[1] R. Rolph and G H Downing

Life Sciences Directorate NASA Ames Research Center Moffett Field California USA and Aviation Medical Research Unit McGill University Montreal Québec Canada

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Abstract Human subjects were vertically oscillated in the dark over a frequency range of 1–0.02 Hz at peak-to-peak acceleration amplitudes of 0.2–0.6 g using both vertical movement simulator and real flight. Subjective tracking of the movement was unreliable and showed no systematic dependence of phase upon frequency In contrast, the oculomotor response, although weak, clearly demonstrated progressive and substantial phase lag with increasing frequency The similarity of this characteristic to that previously obtained from brainstem neural responses in cat suggests the oculomotor response was primarily of vestibular origin The in-flight studies demonstrated that head movement in a changing *linear* accelerative field, especially at 0.1 Hz, is highly provocative of motion sickness despite subthreshold *angular* movement of the aircraft

Previous studies investigating human subjective response to vertical linear acceleration with head erect revealed a peculiar difficulty in assessment of direction of body movement both during sinusoidal motion (Malcolm & Melvill Jones 1974) and during low magnitude step-wise changes in acceleration (Melvill Jones & Young, 1978) These findings were the more surprising in view of the apparent lack of such difficulty in subjects exposed to similar but horizontal linear acceleration (Merry 1966 Young Merry & Li 1966) The combined results of the above authors showed that the directional difficulty cannot be attributed to a specifically low sensitivity to vertically oriented acceleration with head erect. Furthermore there is clearly good directional information in the neural response of the vestibular otolith system to vertical (i.e. predominantly saccular) stimulation at both peripheral (Fernandez & Goldberg 1976) and central (Daunton & Melvill Jones 1973) levels in monkey and cat respectively

Presumably therefore subjective information does not reflect the available neural information in these circumstances Consequently it was decided to compare subjective measures of response with objective measurements of involuntary vertical (sagittal plane) eye movement during exposure of the whole body (seated head erect) to a variety of periodic vertical acceleration stimuli Preliminary experiments investigating the apparent movement of retinal after-images supported the expectation that some form of vertical eye movement would be reflexly induced in these circumstances (McCabe 1964)

METHODS

Eight normal human subjects were exposed to a wide range of sinusoidal vertical accelerations by means of both the NASA Ames Research Center Height Control Test Apparatus (HCTA) and a NASA Lear Jet aircraft as described below Subjective data were obtained from all subjects and oculomotor data from 7 subjects in the HCTA experiments

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[1] Present address: Director Aviation Medical Research Unit, Dept. of Physiology McGill University Montreal, Quebec Canada. This work was conducted at NASA Ames Research Center while Dr G Melvill Jones held Senior Postdoctoral Award from the US National Academy of Science.

Table I *Sinusoidal accelerations employed in the simulator and flight experiments*

Acceleration amplitude is expressed as half the peak to peak amplitude

Period (sec)		1	2	5	10	15	20	30	40	50
Accel Amp (g)	simulator	0.3	0.3	0.3	0.2	0.15	0.1	–	–	–
	flight	–	–	–	–	–	0.3	0.3	[illegible]	0.1

whilst subjective data alone were obtained from five subjects in the flight experiments

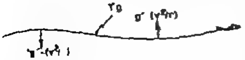
Simulator (HCTA) experiments

The HCTA comprises a small helicopter like cabin running up and down a 100 ft vertical rail and driven by a powerful servo-controlled motor which in turn is under the control of a versatile analogue computer The computer was programmed to drive the HCTA through a series of sinusoidal vertical movements imposing acceleration amplitudes of the largest permissible magnitude (limited by track length) at each of six periodic times ranging from 1 to 20 sec as shown in Table I The minimum magnitude of these amplitudes was at least an order of magnitude higher than subjective threshold levels determined during stepwise changes in vertical linear acceleration (Melvill Jones & Young 1978) Each subject was exposed to a minimum of 20 cycles at each frequency the sequence being rotated from one subject to the next

Subjects were comfortably seated in the simulator cabin with a head restraining harness holding the head in a downward tilted attitude to bring the plane of the horizontal semicircular canals and floor of the utricular maculae close to the true horizontal (Blanks et al 1975) The orientation of the head relative to the true horizontal was routinely adjusted before each run by reference to the outputs of two orthogonally arranged linear accelerometers which were attached to the skull by means of a dental bite board and properly aligned before each experimental series A continuously variable lever was moved smoothly up and down by the subject according to his estimated vertical position in the sequence of vertical movement

Subjects were practised as long as they wished at the 2 and 10 sec per cycle stimuli, whilst being continuously informed about what was happening They were then told that all other stimuli would be of a similar pattern but of varying frequency This policy was adopted rather than incorrectly informing subjects that they would experience random motion for the following reasons First since the stimuli were to be strictly periodic and therefore predictable it seemed important to ensure the results truly reflected the pattern of response to predictable stimuli rather than a hybrid response to quasi-random stimulus patterns as assumed by the subject Secondly if indeed there should be dynamic terms altering the phase of the peripheral sensory response according to stimulus frequency then presumably this should be reflected at least in the involuntary oculomotor reflex whether or not the subject was consciously predicting his sensation

D C electro-oculography (EOG) was employed for recording vertical eye movements bearing in mind the special limitations of this method when recording movements in this direction relative to the head (Barry & Melvill Jones 1965) For this purpose two paired outputs one from each eye were summed and amplified in a local electronic control box All runs and measurements of eye movements were conducted with eyes open in the dark behind blackout goggles after at least 45 minutes dark adaptation to ensure minimal changes in EOG gain (Gonshor & Malcolm 1971) Calibrations were conducted before and after each



[.] 1 Flight path for production of low f equency modulation of vertical g

[.]s through small vertical slits in the goggles [no]rmally closed by black adhesive tape As [ha]d been demonstrated in previous experi[m]ents these brief periods of limited vision [w]ould not be expected to modify EOG gain [A]rousal was of course maintained by continual [su]bjective tracking. Initially additional mental [a]rithmetic was used to further enhance arous[a]l But since this tended to interfere with the [su]bjective tracking task though not appearing [t]o introduce significant changes in oculomotor [r]esponse this procedure was omitted in the [ma]in experiment. Results were recorded both [di]rectly on a multiple channel pen recording [s]ystem and on magnetic tape for further analy[si]s.

Flight experiments

The objective of the flight experiments was to extend the low frequency range of sinusoidal stimulation beyond the simulator limit whilst retaining a substantial stimulus amplitude as shown in Table I Furthermore the flight profile would be relatively free of accessory cues such as track vibration and servo transients at peak accelerations It was intended to include two overlap frequencies of 0 1 and 0 05 Hz. However as described below the 0 1 Hz flight profile produced incapacitating motion sickness so that 0 05 Hz was the only frequency common to both the simulator and flight experiments

The sinusoidal stimuli were generated by executing controlled oscillations in pitch as in Fig 1 whilst flying at a high constant, forward speed and maintaining a constant average altitude To achieve the required stimulus profile an onboard low frequency waveform generator moved a specifically devised cockpit indicator which had to be tracked by means of an adjacent output from a vertical linear accelerometer which was itself located at the aircraft s center of gravity close to the subject It proved necessary to employ two pilots one to maintain juxtaposition of the two indicators by controlling aircraft pitch the other to maintain constant airspeed despite changes in aircraft pitch angle This latter measure served to avoid unnecessary changes of direction in the vertical' g vector by eliminating accelerations parallel to the aircraft s flight path Of course the intended changes in aircraft pitch angle must introduce some directional changes in the *g* vector However such changes could be reduced to a virtually insignificant level by flying at the highest permissible forward speed since for small pitch angles a given vertical acceleration amplitude *A* is associated with a maximum radius of curvature *r* which is directly propotional to the square of the forward velocity V ($A = V^2/r$ see Fig. 1) A simulation study showed that over the whole range of intended stimuli the angular deviation of the linear acceleration vector from true vertical would not exceed 3° and its rate of deviation would not exceed 3 /sec In view of the poor low frequency response of the semicircular canals (e g. Wilson & Melvill Jones, 1979) this range of *angular* velocities would be well below physiological threshold Hence the only significant adequate stimulus would be the vertical' *linear* acceleration which itself would never tilt relative to the true vertical by more than ±3° of arc.

Five subjects were successfully investigated in flight 4 being common to both simulator and flight experiments They were located close to the aircraft s center of gravity The head fixation system and manual indicator for subjective response were the same as those used in the simulator It was intended also to measure eye movement as before all equipment having been standardized for installation in both the simulator and experimental aircraft Unfortunately in the available flight time it did not prove possible to obtain satis-

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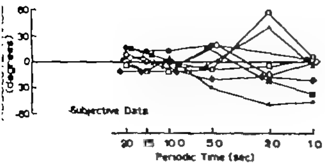

F g 3 Frequency dependence of subjective phase obtained on the simulator

f stimulus. A peculiar difficulty of analysis rose from the fact that on some occasions here a marked correlated response occurred ne could detect something akin to the inter ittent smooth pursuit and saccadic move nents with which one associates compensa ory oculomotor response to sinusoidal rotational stimulation Fig. 5 illustrates the kind of ilemma which can then arise In the trace of ertical eye movement (bottom record) two inds of response pattern can be discerned At he left side of the figure there is the suggestion f a nystagmoid pattern of eye response Thus at the arrow (A) one sees what could be nterpreted as the lower peak of a slow phase ye movement followed at B by the sugges-ion of an upper peak each of these peaks eing situated between two oppositely directed accades as is to be expected if they really were *slow phase* peaks. In the next cycle a omewhat similar situation is seen but with more saccades Further on a faint resemblance is seen but without a clear indication of the same characteristic Superimposed on these phenomena there tended to be a dominant cyclical change in average eye position having the same fundamental frequency as the stimulus

However the phases determined from points A and B are almost the inverse *of those* determined from C and D The question arose which to choose and for what reason? A clear cut answer to this question could not be *found* Consequently each record was taken on *its* own merit, and phase assessments were made on the basis of the largest component of response Indeed It first seemed unlikely that analysis of such *indefinite* data would reveal any meaningful trends Nevertheless, the col lected results in Fig. 4 show that highly meaningful trends *did* emerge with the important feature that these were quite different from

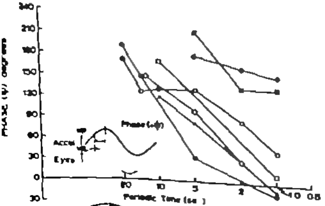

Fig 4 Frequency dependence of vertical oculomotor response

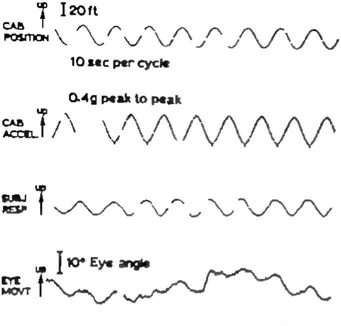

Fig 2 Subjective response and vertical eye movements recorded during sinusoidal variation of vertical acceleration in the NASA Height Control Test Apparatus (HCTA)

factory eye movement records so that the flight results are confined to subjective response data

RESULTS

Simulator experiments

Figure 2 shows an extract of original records of the stimulus (vertical position and acceleration relative to the track) and response (subjective estimate of vertical position on the track and objective record of vertical eye movement) from a simulator run at 0 1 Hz and peak to-peak acceleration amplitude 0 4 g The double differentiation between cab position and acceleration traces reveals some vibration as well as small transients at the moments of changing direction As we shall se below both these acceleration artifacts may well have provided significant accessory subjective cues The records in Fig 2 show a reasonable degree of consistency in both subjective and eye movement records Sometimes however the former showed large variations in phase and the latter only a weakly correlated or even indeterminate signal as described below

Subjective response

Fig 3 plots the mean phase angle between subjective response and actual cab position on a linear ordinate against the periodic time of oscillation on a logarithmic abscissa Each symbol represents the data from one subject and each point the mean value obtained from 20 estimates comprising 10 estimates from the peaks of each of the upper and lower halves of 10 consecutive cycles chosen after the first five cycles of stimulation

Evidently the scatter of phase data was wide especially at the higher frequencies in spite of the detailed knowledge which subjects had of their movement pattern In line with this were numerous recorded voluntary comments such as "I really don t seem to know what s happening" and "How the do you expect me to make a sensible response in these conditions" At lower frequencies the phase estimates were less variable although it should be borne in mind that at these frequencies a given phase error corresponds to a larger error in time

At important feature of the results in Fig 3 is that despite erratic judgements there was no systematic dependence of subjective phase upon the stimulus frequency except perhaps for a weak tendency for the response to phase lag the stimulus at the highest frequencies, probably attributable to the very short delay of around 100 msec associated with this order of lag at these frequencies

Oculomotor response

Figure 4 shows the corresponding data from the analysable records of eye movement in the same set of experimental subjects and runs It may be mentioned here that when initially investigating the oculomotor response records it appeared that the signal if any was so heavily masked by noise—mainly in the form of wandering eye movements—that no useful data would emerge Indeed as will be seen in the figure several data points are missing especially at the low frequency end of the range

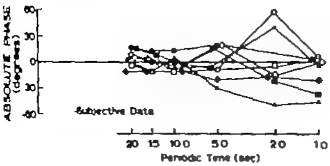

Fig 3 Frequency dependence of subjective phase obtained on the simulator

of stimulus A peculiar difficulty of analysis arose from the fact that on some occasions where a marked correlated response occurred one could detect something akin to the intermittent smooth pursuit and saccadic movements with which one associates compensatory oculomotor response to sinusoidal rotational stimulation. Fig 5 illustrates the kind of dilemma which can then arise In the trace of vertical eye movement (bottom record) two kinds of response pattern can be discerned At the left side of the figure there is the suggestion of a nystagmoid pattern of eye response Thus at the arrow (A) one sees what could be interpreted as the lower peak of a slow phase eye movement followed at B by the suggestion of an upper peak each of these peaks being situated between two oppositely directed saccades as is to be expected if they really were *slow phase* peaks In the next cycle a somewhat similar situation is seen but with more saccades Further on, a faint resemblance is seen but without a clear indication of the same characteristic Superimposed on these phenomena there tended to be a dominant cyclical change in average eye position having the same fundamental frequency as the stimulus

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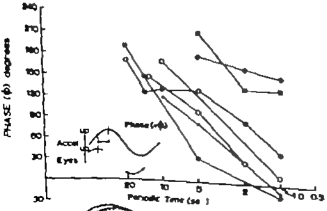

Fig 4 Frequency dependence of 'vertical' oculomotor response

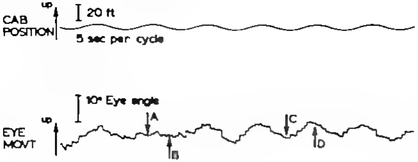

Fig 5 Extract of original recording of vertical eye movements to illustrate problems of data analysis.

those obtained from the subjective response data Thus in Fig 4 a strong tendency is seen for substantial and rapid phase advancement with *decreasing* frequency (i e increasing period) of stimulation In every case where analysis was possible over a sufficient range of frequencies this trend was strongly apparent although there was considerable variability in the absolute level at which this trend appeared to take place It seems reasonable to assume that the variability in absolute level could be attributable to the kind of difficulty in analysis referred to above and illustrated in Fig 5 Indeed it seems likely that the difference between the two records located near the 180 level and the rest of the data in Fig 4 could be due to the phenomenon depicted in Fig 5 Consequently an attempt was made to achieve some form of normalization of the results The outcome of adjusting all values by the amount needed to bring their phase at 1 0 Hz to zero is shown in Fig 6 in which the trend of change in phase with frequency becomes very apparent when compared with Fig 3

Flight experiments

Figure 7 gives the subjective phase data obtained from the flight experiments shown in the same format as the simulator results in Fig 3 As in the simulator data there was no consistent trend of change in phase of response with stimulus frequency There was however much greater dispersion of mean results over the whole frequency range of stimulus and notably without the tendency in Fig 3 for tighter clustering of mean values around zero phase at the lowest frequencies Especially significant is the fact that although 4 subjects were common to both data sets quite different results were obtained in the simulator and flight data at the common frequency of 0 05 Hz

Unfortunately as already mentioned it was not possible to obtain satisfactory eye movement recordings in the time available for the flight experiments

Motion sickness

As mentioned under Methods the flight experiments at 0 1 Hz had to be abandoned due to prohibitive motion sickness especially on

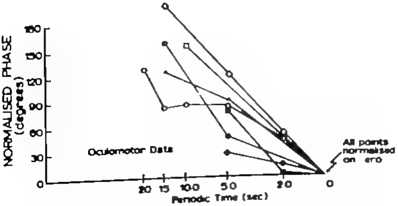

Fig 6 Normalized oculomotor phase data.

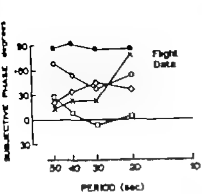

Fig 7 Frequency dependence of subjective phase obtained from in-flight studies.

the part of the in-flight experimenter who had to move his head around during experimental runs in order to monitor and adjust equipment and supervise the subject Although not measured the effect was noticeably less marked as the period of oscillation was increased becoming insignificant at the longest periods of 40 and 50 sec Mild motion sickness was also experienced in the simulator especially at oscillation durations of 5 sec The interesting features of these observations are that the effects occurred in the absence of significant angular movement and that the incapacitating effect in flight was confined to the moving experimenter the significance of which is discussed below

DISCUSSION

The results of subjective estimates in these experiments are in line with both earlier (Malcolm & Melvill Jones 1974) and subsequent (Watt, 1977) observations indicating that *subjective* tracking of vertical motion with head erect is unreliable and on average shows no dependence of phase upon the frequency of sinusoidal stimulation The scatter of the present simulator results was however less than that of the previous ones a difference probably attributable to the larger stimulus amplitudes employed here and the fact that our subjects were fully informed of the sinusoidal stimulus pattern and were well practised in its recognition The subjective flight data also showed additional similarities to those of the previous in-flight (helicopter) experiments in sofar as both sets of in-fight data produced wider scatters of data points than those of the corresponding simulator tests Furthermore the tendency to better resolution of phase with decreasing frequency seen on the simulator was not evident in flight.

In contrast to the subjective results of Figs 3 and 6 the *reflex oculomotor* results of Figs 4 and 6 show a strong dependence of phase upon frequency This could hardly be accounted for by known oculomotor dynamics (Skavenski & Robinson 1973) owing to the short duration of the relevant oculomotor time constant (0 16 sec) On the other hand comparison of Fig. 6 with the previously reported neural data of Fig. 8 (Melvill Jones & Milsum 1969) obtained from cat brainstem neural units during oscillatory linear acceleration suggests rather strongly that the involuntary oculomotor response followed in a general way some component of the likely brain stem neural signal That this would be neurophysiologically feasible is indicated by the presence of known neural connections be

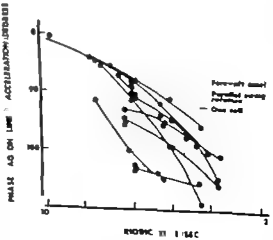

Fig 8 Phase data obtained from vestibular neural units in cat brainstem (reproduced from figure 6 of Melvill Jones & Milsum 1969).

tween saccular afferents and the oculomotor system (Fluur & Mellstrom 1970 Hwang & Poon 1975 Precht et al 1979) and the fact that there is an abundance of highly sensitive saccular linear accelerative information in the primary afferent vestibular signal (Fernandez & Goldberg 1976) It is interesting in this context that a preliminary report on H reflex testing of vestibulo-spinal during sinusoidal vertical acceleration did not note frequency dependent phase changes in the modulation of motoneurone pool excitability in the lower lumbar region of the cord (Watt 1977)

A feature of these oculomotor data was that despite a clear phase characteristic in the ensemble results the periodic content of individual records was often weak and fluctuating This is in accord with the results of Niven Hixson & Correia (1966) who found negligible oculomotor response to sinusoidal linear acceleration along the Z-axis of the head as compared with that induced by side-to-side movements Again Vidic et al (1976) reported much worse ocular fixation on an *imagined* stationary point during vertical than during horizontal oscillation Despite these latter reports it should be recalled however that McCabe (1964) demonstrated distinct vertical eye movements during whole body vertical oscillation although their dynamic response characteristics were not quantitatively analysed

If as the above discussion suggests the oculomotor data reflects in some measure the vestibular input then the absence of phase in the subjective response would seem to suggest the latter was due primarily to accessory somatic cues rather than the vestibular signal But if this is true why then do we not see an element of phase in the flight data when changes of vibration and acceleration transients associated with the simulator are no longer present? Presumably the additional somatosensory cues from continually changing pressure on the seat and harnesses together with internal visceral cues are accepted in preference to the vestibular input The unusually prolonged patterns of these stimuli together with the well known adaptive characteristics of such sensory inputs could then account for the gross subjective uncertainty indicated by these and the previous helicopter experiments

It is interesting to compare the present subjective responses with those of Young Meiry & Li's subjects (1966) exposed to sinusoidal saccular stimulation on a horizontal linear accelerator Unlike our findings they reported marked dependence of phase (not unlike that of Figs 6 and 8) in their tracking response as a function of stimulus frequency This difference between vertical and horizontal response is consistent with the similar finding that whereas supine subjects exposed to near threshold horizontal stimuli of a stepwise nature could reliably assess their direction of movement (Meiry 1966) subjects seated erect and exposed to similar vertical stimuli could not (Melvill Jones & Young 1978) Thus the difference seems well substantiated although difficult to explain Possibly the changing direction of the resultant gravito-inertial vector which is inevitably associated with horizontal but not vertical linear acceleration could account for the difference

Whatever the cause the grossness of directional uncertainty during vertical movement carries a message of caution in the applied context of aviation and emphasises the need for clear and unambiguous instrument indication of vertical movement in VTOL aircraft especially since direct vision provides no useful aid in this respect when flying at altitude (Malcolm & Melvill Jones 1974)

If the difference between the data in Figs 3 and 6 does indeed reflect large discrepancies between general somatosensory and vestibular afferent information then this discrepancy could well account for the incidence of motion sickness found in these experiments due to sensory conflict (Money 1970) However there is an additional important feature introduced by the fact that incapacitating sickness only occurred in the experimenter who

was moving about in the flight experiments. Even when the subject and experimenter changed roles during a given flight it was still the experimenter not the subject who became incapacitated. It is of course well known that head movements during turbulent flight provoke nausea whilst the opposite effect is associated with head fixation (Johnston & Mayne 1953; Money 1970). However it is often assumed that the special nauseating stimulus of head movement derives from coriolis cross-coupling effects in the canals. Yet in the present experiments aircraft *angular* movement would have been subliminal as would the attendant *canal* coriolis stimuli. Consequently it seems likely that the especially provocative stimulus here was due to head movement relative to a *linear* accelerative field, which although changing in magnitude was not itself subject to significant change of direction. Perhaps when the otolith dynamic response is as well understood as that of the canals an explanation will emerge which is somewhat analogous to that accounting for coriolis cross-coupling errors in the canals (e.g. Melvill Jones 1970) associated with *angular* head movements in the presence of low frequency *rotational* acceleration.

ZUSAMMENFASSUNG

Versuchspersonen wurden im Dunkeln vertikal über einen Frequenzbereich von 1–0,02 Hz in Schwingung versetzt bei Minimum-zu-Maximum-Beschleunigungsamplituden von 0,–0,6 g. Vertikalbewegungssimulatoren sowohl als tatsächlicher Flug wurden angewendet. Subjektives Verfolgen des Bewegungsablaufs war unzuverlässig und hat keine systematische Abhängigkeit der Phase von der Frequenz gezeigt. Demgegenüber hat die oculomotorische Reaktion, obwohl sie schwach war, doch klar progressive und beträchtliche Phasenverschiebung mit zunehmender Frequenz erwiesen. Die Ähnlichkeit dieses Charakteristikums mit dem, was früher bei neuralen Reaktionen im Hirnstamm der Katze beobachtet wurde, läßt darauf schließen, daß die oculomotorische Reaktion hauptsächlich vestibulärer Herkunft war. Die „Im-Flug"-Studien haben gezeigt, daß Kopfbewegung in einem wechselnden Beschleunigungsbereich, besonders bei 0,1 Hz, stark zu Kinetose Anlaß gibt, trotz unterschwelliger Winkelbewegung des Flugzeuges.

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tween saccular afferents and the oculomotor system (Fluur & Mellstrom 1970 Hwang & Poon 1975 Precht et al 1979) and the fact that there is an abundance of highly sensitive saccular linear accelerative information in the primary afferent vestibular signal (Fernandez & Goldberg 1976) It is interesting in this context that a preliminary report on H reflex testing of vestibulo-spinal during sinusoidal vertical acceleration did not note frequency dependent phase changes in the modulation of motoneurone pool excitability in the lower lumbar region of the cord (Watt 1977)

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If as the above discussion suggests the oculomotor data reflects in some measure the vestibular input then the absence of phase in the subjective response would seem to suggest the latter was due primarily to accessory somatic cues rather than the vestibular signal But if this is true why then do we not see an element of phase in the flight data when changes of vibration and acceleration transients associated with the simulator are no longer present? Presumably the additional somatosensory cues from continually changing pressure on the seat and harnesses together with internal visceral cues are accepted in preference to the vestibular input

The unusually prolonged patterns of these stimuli together with the well known adaptive characteristics of such sensory inputs could then account for the gross subjective uncertainty indicated by these and the previous helicopter experiments

It is interesting to compare the present subjective responses with those of Young Meiry & Li's subjects (1966) exposed to sinusoidal saccular stimulation on a horizontal linear accelerator Unlike our findings they reported marked dependence of phase (not unlike that of Figs 6 and 8) in their tracking response as a function of stimulus frequency This difference between vertical' and horizontal' response is consistent with the similar finding that whereas supine subjects exposed to near threshold horizontal stimuli of a stepwise nature could reliably assess their direction of movement (Meiry 1966) subjects seated erect and exposed to similar vertical stimuli could not (Melvill Jones & Young, 1978) Thus the difference seems well substantiated although difficult to explain Possibly the changing direction of the resultant gravito-inertial vector which is inevitably associated with horizontal but not vertical linear acceleration could account for the difference

Whatever the cause the grossness of directional uncertainty during vertical movement carries a message of caution in the applied context of aviation and emphasises the need for clear and unambiguous instrument indication of vertical movement in VTOL aircraft especially since direct vision provides no useful aid in this respect when flying at altitude (Malcolm & Melvill Jones 1974)

If the difference between the data in Figs 3 and 6 does indeed reflect large discrepancies between general somatosensory and vestibular afferent information then this discrepancy could well account for the incidence of motion sickness found in these experiments due to sensory conflict (Money 1970) However there is an additional important feature introduced by the fact that incapacitating sickness only occurred in the experimenter who

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PERCEPTUAL AND ACOUSTIC CORRELATES OF ABNORMAL VOICE QUALITIES

Britta Hammarberg, B Fritzell J Gauffin J Sundberg[1] and L Wedin[2]

From the Department of Logopedics and Phoniatrics Huddinge University Hospital, Huddinge Sweden

(Received January 3 1980)

Summary A set of 17 voices representing various voice disorders were presented to a jury of voice experts who rated them on a 5-point scale for each of 28 terms frequently used for describing voices. A statistical treatment of these ratings yielded 5 bipolar factors accounting for 85% of the total variance. Significant correlations were found between four of these factors and certain acoustic data extracted from long time-average spectra and fundamental frequency distribution analysis of the same voice sample

In logopedics and phoniatrics where examination and evaluation of results are usually based on subjective observations and judgements there is a great need for objective methods that will give numerical measurements While a number of methods for acoustic analysis have been developed for research purposes clinical applications have been slow in materializing. Many of the patients that we see at the Department of Phoniatrics Huddinge University Hospital have voice problems During the five year period 1974–78 47% of the new patients admitted to the Department were voice patients

In order to supplement our subjective examination of the voice characteristics of our patients we have begun a search for relevant acoustic methods of analysis These would serve as an aid in diagnosis and in the evaluation of therapy progress The clinically well trained ear will always be the primary and most important means of examination however For this reason the first part of our study has been a perceptual analysis of voice characteristics and the results herefrom have then been used for comparison with acoustic data.

Perceptual Voice Analysis

In phoniatric and logopedic clinics in Sweden a more or less firmly established set of terms is in use to describe voice qualities. These terms are based on impressionistic and perceptual judgements Little is known about the connections between terms describing voices In a study of the results of voice therapy Lundålv & Nygren (1974) found that logopedists and students of logopedics used 88 different terms for the description of voice quality and pitch in a sample of 10 patients

Our approach in this investigation was designed to analyse the correlations between voice-describing variables used in our clinic in order to structure the terminology and give a more precise frame of reference for voice perception by the statistical method of factor analysis The perceptual qualities of human voices can be interpreted as bundles of various traits or characteristics which are more or less closely connected with each other By means of factor analysis one can obtain a limited number of clusters of variables with high intercorrelations Such clusters are then treated as factors

Factor analysis has been used previously for studies of voice perception and acoustics (Isshiki et al 1969; Isshiki & Takeuchi 1970

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G Melvill Jones F.R S
A viation Medical Research U it
Rm 1223 McIntyre Med Sc Build
3655 Drummond street
Montreal H3G 1Y6
Canada

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Table I The voice-describing variables in Swedish and their approximate equivalents in English

Swedish professional term	English equivalent
1 läckande	breathy
2 skrovlig	rough raucous
3 diplofoni	diplophonia
4 knarr	creaky vocal fry
5 halsig	guttural throaty
6 återhållen	repressed/restrained
7 grov	coarse
8 pressad	strained
9 strypta frasslut	strangled phrase endings
10 klangfattig	lack of timbre
11 afoniska inslag/afoni	moments of aphonia/aphonia
1 väsande	wheezing hissing
13 bitonalitet	bitonality
14 hyperkinetisk	hyperfunctional
15 monoton	monotonous
16 beslöjad	husky
17 klangförändring utan brott	quality changes without voice breaks
18 fladder	flutter
19 skrap	grating
20 registerbrott/ registerbrottstendens	voice breaks/ tendency towards voice breaks
21 hårda ansatser	hard glottal attacks
22 instabil klang	unstable quality
[illegible] strav	harsh
24 bröstregister	chest register
25 mellanregister	middle register
26 falsettregister	head (falsetto) register
27 röstläge	pitch
28 instabilt röstläge	unstable pitch

Takahashi & Koike 1975) In these studies the stimulus consisted of sustained vowels From a clinical point of view however it is not satisfactory to attempt to evaluate voice quality from a sustained vowel alone Changes in *running speech such as vocal onset* and termination voice breaks etc are crucial to voice quality and are not likely to appear in a single vowel sound In our study the stimulus material consisted of a reading of a standard text of approximately 40 sec duration

In the three studies mentioned above Osgood's semantic scaling method was used In this case the input terms consist of bipolar verbal scales such as awful–nice sweet–sour soft–hard which are commonly used adjectives with no special reference to voice perception

METHODS AND MATERIALS

Words selected to suitably describe perceptual impressions were selected from terms actually used by clinicians in our clinic to describe 115 individual voice evaluations in patient case histories The 28 most frequently used terms were selected from a total of some 50 terms. Of these 23 refer to *voice quality* 2 to *pitch* and 3 to *register* These terms with English equivalents are listed in Table I

The stimulus consisted of a short story of 92 words read into a tape recorder by 17 subjects with various functional and organic voice disorders including vocal nodules (2) chronic laryngitis (2) unilateral paralysis (2) polyps and polypoid thickening of the vocal cords (4) contact ulcer (1) laryngeal cancer (1) incomplete mutation (1) and functional dysphonia (4) Three of the voices were duplicated in order to estimate the consistency of the listeners so that the listening tape included 20 voice examples There were 8 male patients and 9 female patients ranging in age from 17 to 70 For the listening test a 28-variable test form was prepared The listeners were the staff members of the phoniatric department of Huddinge Hospital i e one male phoniatrician and 13 female logopedists Each voice example was replayed four times and a short passage of music was inserted between the voice examples to distract the listeners The listeners rated their auditory impression of the voices on a 5-point scale for each of the 28 variables

The tape recordings were made in a soundproof room with a two-channel tape recorder (Revox A77) which has stepwise variable gain controls One channel records the signal from a small microphone mounted on a spectacle frame worn by the speaker (in order to keep a constant mouth-to-microphone distance) The other channel records the signal from a contact microphone attached to the skin below the cricoid cartilage Information of the sound pressure level (SPL) of the speech is preserved by the recording of a reference tone and by the use of a stepwise

Table II *The proportion of variance explained by the five factors*

Factor	1 Unstable Steady	2 Breathy–Overtight	3 Hyper Hypofunctional	4 Coarse Light	5 Head–Chest register
Variance explained	30 0%	27.3%	13.5%	10 1%	4 4%

amplifier control and a fixed mouth-to-microphone distance

Results of Perceptual Analysis

The correlations of the mean ratings of all the 28 variables over the 20 voices were tabulated in a correlation matrix The Principal Component Analysis (Harman 1967) was used Factor analysis produced five factors with latent roots larger than 1 0 The five significant factors were rotated to a varimax criterion. The variance explained by each factor is presented in Table II and five factors explained 85.3% of the variance

The factors all turned out to be bipolar The significant factor loadings of the variables are presented in Table III

The most important factor identified by the factor analysis has been labelled factor 1 *unstable–steady* because it included the variables bitonality diplophonia, unstable pitch flutter grating voice breaks and unstable quality which all loaded highly negatively on this factor The only variables with significant positive loadings were restrained' at the 1% level and chest register at the 5% level The heavy load in the negative pole of the factor seems to reflect the pathological voice sample while the positive pole stands for a more *normal* voice quality for which neither variables nor voice examples are present in this experiment. As can be seen from Table I only two out of 26 variables (no 24 chest register' and no 27 pitch) refer to normal voice function while the rest refer to pathological voice function

Factor 2 included the variables 'breathy wheezing lack of timbre moments of aphonia and husky all negatively loaded opposed to the positively loaded creaky/vocal fry' (Sw *knarr*) The variables in the negative pole have a clinically recognizable common trait of breathiness contrasted to overtightness in creaky/vocal fry Factor 2 therefore was labelled *breathy–overtight*

The character of *factor 3* was strained–strangled' opposed to monotonous–restrained' The positively loaded variables were strangled phrase endings strained hyperfunctional guttural/throaty and glottal attacks Negatively loaded were monotonous and husky We interpreted factor 3 as *hyperfunctional–hypofunctional*

Positively loaded in *factor 4* were the variables coarse rough and harsh contrasted to high pitch middle register and restrained' which loaded negatively This factor reflected a 'harsh–dark quality towards a *high–light* which made us label factor 4 *coarse–light*

Factor 5 was easily described as it turned out to consist of only one variable in each pole *head register* versus *chest register*

As a final step in the factor analysis, factor scores were calculated for each of the 20 voice examples (including the three duplicated ones) Table IV The factor scores give the position of each voice on each of the five factors. The factor scores were then correlated with the results of the acoustic analysis.

In order to test the consistency of the listener s ratings the retest reliability was calculated for the first and second presentations of the three duplicated voices (i.e voices no 3/11 6/14 and 12/18) Pearson s correlation coefficient was calculated on the mean ratings of the 28 variables for each paired voice The correlation coefficients were all very high, 0 93–

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Table IV *Factor scores of the 20 voice examples including the three duplicated voices (nos 3=11 6=14 12=18)*

For example voice no. 9 has high negative score in factor 2, which means high degree of 'breathiness or voice no 17 with high positive score in factor 4 a high degree of coarse/rough

Voice	Factor scores Factor 1	Factor	Factor 3	Factor 4	Factor 5
1	−0 865	0.979	+0 017	+0 40	+0 100
	+0 084	−0 049	−1.323	−0 150	+0.404
3	−0 815	−0 067	+0.291	+1 710	+0 275
4	+0 697	0.965	−1.680	−1.287	+1.071
5	−0 971	+0 033	−0.558	−1.356	−0 876
6	+0 652	+0 072	+0.365	−0.209	−0.251
7	−0.164	−1 147	+0 735	−0 354	−0 863
8	+1.051	+1 093	−0 401	+1.252	0 004
9	+0 507	$\boxed{-2.575}$	−0 677	+0.075	−2 068
10	+0 157	1.040	+0.684	+1.098	0 618
(11	−0.239	0.186	+0.199	+1 437	−0.310)
12	0 858	−0 452	+...11	−0 462	+1 107
13	−0 682	+1 663	+1.246	−0.827	2.515
(14	0 661	−0 109	+0.045	−0 765	−0 072)
15	+0 689	+1 557	−0.281	−1.269	+0.574
16	−2.417	−0.240	−0.593	−0 036	0.524
17	1 097	0.746	−1.056	$\boxed{+1.947}$	0 470
(18	+0 757	−0 349	+1.896	−0.348	+0.933)
19	1 937	−0 157	+0 010	0 006	+1.394
20	+0 879	0 816	−1 035	−0 671	+0 277

frequencies of 0 2 5 and 8 kHz, and the maximum level was determined in each band The maximum level of the 0–2 kHz band corresponds to the averaged overall sound pressure level (SPL) of the voice The peak level differences between the three bands were then determined These values plus the mean pitch frequency (determined from the FFDA) were correlated with results from the perceptual analysis

Perceptual–Acoustic Comparison

Multiple regression analysis was used to correlate the factor scores of the five perceptual factors with the acoustic data The acoustic variables were put into the regression equation one by one in rank order of explained variance (stepwise multiple regression) Multiple regressions for the maximum correlation between the acoustic variables and each of the five factors is shown in Table V

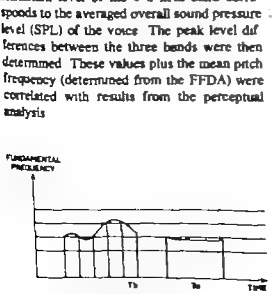

Fig 1 ( ) Simplified figure showing how fundamental frequency histogram is calculated from the fundamental frequency as function of time The frequency area is divided into number of intervals b

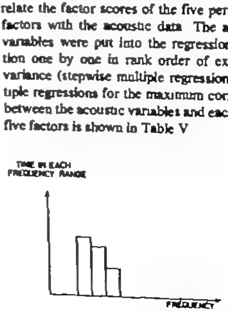

Fig 1 (b) The total time in each interval is measured and plotted in this figure

Table III *Ranking of the significant factor loadings of the variables (>0.50) in each of the 5 factors*

All loadings are significant at the 1% level except those within braces which are significant at the 5% level

Factor 1 Unstable–Steady	
+.535	restrained
[+.377	chest register]
−.669	changes of voice quality
−.774	unstable quality
−.774	voice breaks
−.795	gratings
−.810	flutter
−.827	unstable pitch
−.831	diplophonia
−.877	bitonality
Factor 2 Breathy–Overtight	
+.787	creaky vocal fry
−.730	husky veiled
−.773	intermittent aphonia
−.832	lack of timbre
−.849	wheezing
−.899	breathy
Factor 3 Hyper–Hypofunctional	
+.857	strangled phrase endings
+.831	strained
+.81	hyperfunctional
+.698	guttural throaty
+.540	hard glottal attacks
[−.364	husky]
−.675	monotonous
Factor 4 Coarse–Light	
+.930	coarse
+.858	rough
+.701	harsh
−.618	restrained
−.671	middle register
−.760	high pitch
Factor 5 Head–Chest register	
+.577	chest register
−.747	head register

0.97 This means that the consistency of the listeners is statistically reliable

To test factor validity i e to see if the factor analysis really produced factors relevant to the perception of voice quality a second listening test with the same 20 voice examples was given The same listeners as before (except for one drop-out) rated the voices on a 9 point scale for each of the five factors The contents of the factors as shown in Table IV had been explained to the listeners The mean values for all listeners per factor were correlated with the factor scores of the voices from the first listening test The correlations (Pearson s *r*) were all significant at the 1% level for the first four factors varying from 0.80 to 0.91 for factor 5 registers 0.60 The validity of these factors can therefore be said to be very good

Acoustic Analysis

Two acoustic methods were used fundamental frequency distribution analysis (FFDA) and long-time average spectrum analysis (LTAS) both of which are capable of using tape recorded speech for analysis

The FFDA uses the signal from the contact microphone (see above) and is performed by a computer with the result given as a histogram (Figs 1 and 2) The computer program also calculates the most common frequency the mean value of the fundamental frequency and a straight-line approximation of the distribution The program is a modification of a program used for analysis of music (Askenfelt 1976) and gives a more detailed analysis than needed for the present purpose

For the LTAS another program for music analysis (Jansson & Sundberg 1976) was used Speech material of approx 40 sec duration is fed through a number of filters dividing the frequency range into 51 channels 250 Hz wide The level of each channel is averaged by the computer and is plotted on a frequency–intensity diagram (Fig. 3) All voiceless speech sounds were automatically eliminated by means of the sound energy in the lowest frequency bands

The filterbank and computer program gives a very detailed LTAS and the equipment is probably too elaborate to be used in routine diagnosis (Fig 4) This is a research tool and we aim at designing simple and easy to handle clinical instruments in the future

The LTAS curves were evaluated as illustrated in Fig 5 Each curve was divided into three separate frequency bands limited by the

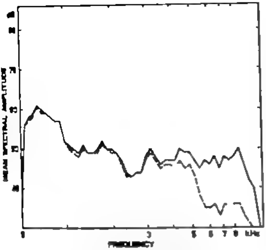

Fig 4 A long-time-average spectrum, LTAS as plotted by the computer 51 band-pass filters have been used The mean output from each filter is plotted as point and each point is connected by straight line The continuous curve is derived from the complete microphone signal, while the dashed curve presents signal from which most of the consonants have been excluded

Hyperfunctional' voice quality showed a significant correlation with high spectral level in all the three LTAS frequency bands but the SPL in the frequency band 2–5 kHz turned out to be the most important one explaining 53%

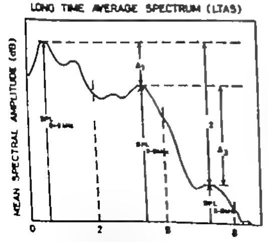

Fig 5 Schematic illustration of the measures extracted from the long-time-average spectra

Table V *Results of the stepwise multiple regression analysis*

The % variance xplained by factors 3 and 5 is fully acceptable (47%, 60% and 57% respectively) while factor 1 is not satisfactorily xplained by acoustic data. For factor 4 significant correlation with acoustic data (MF$_4$) was obtained only for subgroups of the voices see further in the text

Perceptual factor	Acoustic variable entered	Multiple correlation ( )	% explained variance (r^2)
Factor 1			
Unstabl Steady			
Step 1	MF	.37	14
Step 2	SPL–SPL	43	20
Step 3	SPL	48	23
Factor 2			
Breathy–Overtight			
Step 1	(SPL–SPL) (SPL–SPL)	.59	35
Step 2	MF	69	47
Factor 3			
Hyper–hypo-functional			
Step 1	SPL	73	53
Step 2	MF	78	60
Factor 4			
Coarse–Light			
Step 1	MF	.39	15
Step 2	SPL–SPL	.50	25
Factor 5			
Head–Chest register			
Step 1	MF	.63	39
Step	SPL–SPL	75	57

of the factor variance (Fig 7*a*) Hypofunctional' voice was characterized by the same spectrum as breathy in factor 2 ($r=0.56$) (Fig 7*b*). In addition high MF explained another 7% of the factor variance

Factor 4 Coarse–light' The coarse portion of this factor consisted of the variables coarse–rough–harsh while the opposite light was made up of the combination high pitched–restrained–mid-register' Hence this factor would depend not only on timbre but also on the mean pitch frequency A significant correlation existed between Factor 4 and MF

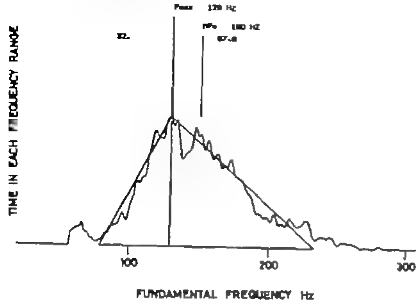

Fig 2 A fundamental frequency histogram as plotted by the computer In this case each frequency interval is only 1 Hz and the histogram appears as a continuous curve

Factor 1 Unstable–steady No significant correlation was found between factor I and any of the acoustic variables This is not surprising as this factor can be assumed to reflect fluctuations in voice quality and the acoustic data give only average signal characteristics

For factor 2 *Breathy–overtight* there was a significant correlation between the breathy voice and the slope of the LTAS curve A steep fall of the spectral level from the frequency band 0–2 kHz to the frequency band 2–5 kHz is evident while the spectral level in band 5–8 kHz was almost at the same level as the spectral level in band 2–5 kHz (Fig 6*a*) This acoustic variable explained 35% of the total variance Overtight ('vocal fry/creaky') on the other hand was characterized by a steep fall in the spectral level from the frequency band 2–5 kHz to the frequency band 5–8 kHz ($r=0.55$) (Fig 6*b*) This is not shown in Table V but the correlation is interesting compared with the LTAS for a breathy voice Another 12% of the total variance was explained by the mean fundamental frequency (MF) i e overtight ('vocal fry/creaky') correlated with low MF ; breathy with high MF

Factor 3 Hyper–hypofunctional This factor relates to vocal effort in the broad sense

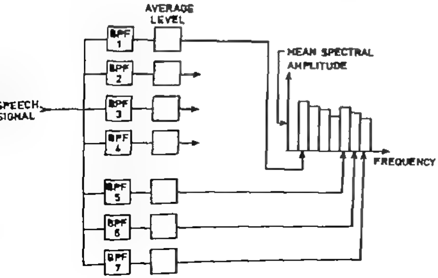

Fig 3 Block diagram showing how the long-time-average spectrum LTAS is derived from the speech signal by using in this case seven band-pass filters

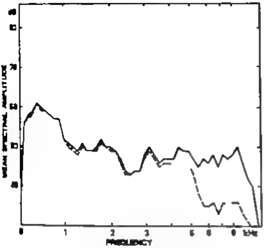

Fig. 4 A long-time-average spectrum, LTAS as plotted by the computer 51 band-pass filters have been used The mean output from each filter is plotted as point and each point is connected by straight line The continuous curve derived from the complete microphone signal, while the dashed curv present signal from which most of the consonants have been xcluded

Hyperfunctional' voice quality showed a significant correlation with high spectral level in all the three LTAS frequency bands but the SPL in the frequency band 2–5 kHz turned out to be the most important one explaining 53%

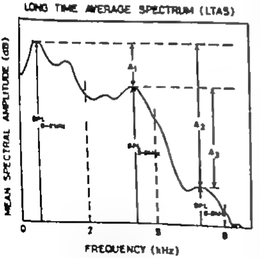

Fig. 5 Schematic illustration of the measures extracted from the long-time-average spectra.

Table V *Results of the stepwise multiple regression analysis*

The % variance xplained by factors 2, 3 and 5 is fully acceptable (47%, 60% and 57% respectively) while factor 1 is not satisfactorily xplained by acoustic data For factor 4 significant correlation with acoustic data (MF$_s$) was obtained only for subgroups of the voices' see further in the text

Perceptual factor	Acoustic variable entered	Multiple correlation ( )	% explained variance (r^2)
Factor 1			
Unstable Steady			
Step 1	MF	.37	14
Step	SPL[illegible]–SPL[illegible]	45	20
Step 3	SPL[illegible]	48	23
Factor 2			
Breathy Overtight			
Step 1	(SPL[illegible]–SPL[illegible])		
	(SPL[illegible]–SPL[illegible])	.59	35
Step 2	MF	69	47
Factor 3			
Hyper–hypofunctional			
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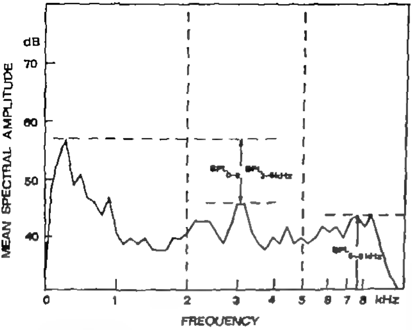

Fig 6 (a) LTAS from a voice with high negative factor score in factor i.e a *breathy* voice (voice no. 9 female unilateral paralysis)

only in female voices and in a subgroup of the male voices with normal SPL

Factor 5 Head–chest register As was expected there was a highly significant correlation between the MF and this factor i e high MF – head register This acoustic variable explained 39% of the total variance Chest register also showed a significant correlation with loss of spectral energy in the upper frequency band 5–8 kHz of the LTAS (18% of the total variance)

DISCUSSION

By means of factor analysis we have shown that in a sample of pathological voices 28 clinical voice-describing variables could be analysed as to their interrelationships and reduced to a simpler structure of five bipolar factors The factors were clinically valid and the reliability of the listener group was very good The factor analysis also showed interrelationships between the voice-describing

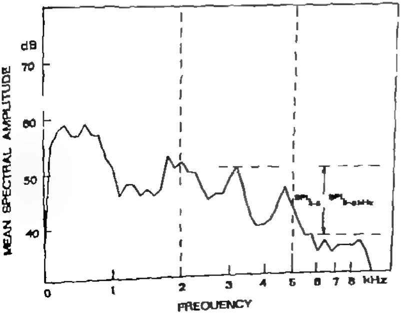

Fig 6 (b) LTAS from voice with high positive factor score in factor i e *over tight (creak vocal fry)* voice (voice no 15 female vocal nodules)

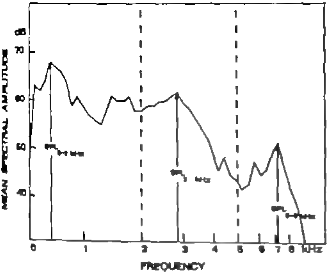

Fig 7 (a) LTAS from voice with high positive factor score in factor 3 1 *hyper functional*" voice (voice no 12 male laryngeal tumour)

variables (Table III) which we had not been aware of until now This clustering of variables with high correlations within individual factors seems to be one way of systematizing the terminology for perceptual voice description Some of the variables obviously reflect the semantic similarities of the terms used The extension of these investigations with larger voice samples and listener groups is in progress and might eventually yield a useful perceptual instrument for voice analysis

Four of the five perceptual factors correlated with acoustic data from the LTAS and the FFDA This indicates that the two methods of acoustic analysis are useful for differentiating voice qualities and for measuring pitch. As pointed out above however both methods were primarily designed for analysis of music

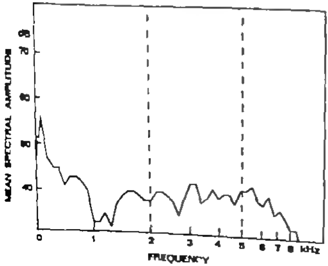

Fig 7 (b) LTAS from voice with high negative factor score in factor 3 *Le hypofunctional*" voice (voice no 4 male, unilateral paralysis).

at the laboratory level and the research equipment used is much too elaborate for the clinic Therefore a modification and simplification of the instruments for the clinical purpose is important

Similar methods of LTAS analysis have been explored by Frøkjaer Jensen and his collaborators (Frøkjaer Jensen & Prytz, 1974) One attempt to find characteristic LTAS curves for three different voice disorders (unilateral paralysis chronic laryngitis vocal nodules) was made by Prytz (1977) He compared the pathological material with normal voices by using computer averagings of the total energy spectrum of the LTAS No characteristic LTAS curves could be obtained so the conclusion was that LTAS cannot be used as a diagnostic instrument These findings neglect the fact that patients with the same diagnosis may differ greatly in voice quality Furthermore Prytz did not separate the consonants from the vowels in the LTAS analysis which means that high frequency noise of the voice source could not be differentiated from the voiceless speech sounds

In order to determine the fundamental frequency the signal from a contact microphone was used Askenfelt et al (1977) found that an electroglottograph was somewhat more accurate However the difference was negligible for clinical purposes and the contact microphone has the advantage of being simpler to handle in the clinic

To obtain a representative sample of voice qualities and to include most of the perceptual cues which contribute to the general impression of a voice connected speech of 40 sec duration was analysed This restricts the choice of acoustic methods For example perturbations of frequency and amplitude i e variation in the length of periods or in the amplitude of succeeding glottal waves are known to occur with hoarseness but this kind of measure has previously been used for sustained vowel analysis only (Wendahl 1963 1966 Koike et al 1977) In our work in progress we have included a method to measure frequency perturbation which can be used for connected speech

ACKNOWLEDGEMENT

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ZUSAMMENFASSUNG

Tonbandaufnahmen von 17 Patienten mit verschiedenen Stimmstörungen wurden von einer Gruppe von Stimmpädagogen (Logopädinnen und Phoniatern) abgehört und beurteilt. Dabei wurde ein 5stufiger Maßstab mit je 28 klinisch geläufigen Termini für verschiedene Stimmeigenschaften angewandt Die statistische Auswertung dieser Hörerurteile ergab 5 bipolare Faktoren die 83% der gesamten Varianz deckten Signifikante Korrelationen ergaben sich zwischen vier der Faktoren einerseits und gewissen akustischen Daten andererseits die durch statistische Langzeitanalysen sowie Analysen der Grundfrequenzverteilung desselben Stimmsamples ermittelt worden waren

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Britta Hammarberg
Dept of Logopedics and Phoniatrics
Huddinge University Hospital
S-141 86 Huddinge
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	Age (years)	Smoking (years)	Exposure (years)
Exposed group			
1	44	26	[illegible]
	45	1	12
3	31	12	9
4	60	42	4
5	[illegible]	–	13
6	64		29
Control group			
1	46	27	
	41	25	
3	45	3	
4a	65	45	
4b	68	45	
5a	31		
5b	37		

By taking a careful history the duration and intensity of different symptoms from the respiratory tract as well as the smoking habits were noted Changes from normal were evaluated in a clinical examination of the nose nasopharynx and lungs The nasal mucociliary function was estimated according to the saccharine test described by Andersen et al (1974) *measuring the time lapse for a methylene-coloured saccharine particle with a diameter of 1 mm to move through the nose* from a point ½ cm behind the anterior edge of the inferior turbinate until the patient sensed a distinct sweet taste and the blue colour was observed in the nasopharynx Room temperature was 23°C and the relative humidity 21–33.5%

Biopsies were taken under local anesthesia from the mucosa of the inferior turbinate 1 cm behind its anterior edge The biopsy specimens were fixed in 10% neutral-buffered formalin and embedded in paraffin cut at various levels and stained with haematoxylin and eosin Small pieces of the biopsies were also fixed in osmium tetroxide for 2 hours These specimens were then embedded in Epon and cut with an ultramicrotome at 1 μm and stained with warm toluidine blue

For the exposed group the test battery also included spirometry measuring FEV VC FVC X-ray of the sinus and pulm culture from the nasopharynx ESR, WBCC eosinophils IgE and RAST-analysis of dust, cladosporium alternaria and housedust mite

RESULTS

The exposed men had worked for 4–29 years (mean 15 years) as lathe operators The histories disclosed nasal symptoms in 5 of 6 workers, starting some years (1–14 mean 7) after their first occupational exposure to oil mist. Three of them had a marked relation to time of work getting better during holidays The two workers with the most pronounced symptoms had been treated earlier for recurrent sinuitis diagnosed by X-ray Only one worker suffered from a cough with expectorate and conjunctivitis beside his nasal symptoms There was no history of allergy except in one subject whose nasal symptoms were accentuated in early spring

The clinical examination of the patients revealed grossly normal nasal mucosae A minor polyp was found in one nose and mucopurulent *secretions were found in three others* One patient had a pronounced deviation of the septum probably due to a trauma 20 years earlier however the history of nasal blockage was only 5 years In the controls the histories and examinations revealed no pathology

The mucociliary function did not differ markedly between the two groups according to the saccharine test. The flow rate is said to be about 0 46 cm per min, with very wide ranges The clearance time can thus be expected to average 20 min In both the exposed group and the control group there were 2 subjects with high values as a sign of reduced function (Table II)

All histological slides were examined on several occasions and without any knowledge of neither the clinical history or of previous exposure to oil mist The results are summarized in Table III

Acta Otolaryngol 90 452–459 1980

UPPER AIRWAY PROBLEMS IN INDUSTRIAL WORKERS EXPOSED TO OIL MIST

K Irander H B Hellquist Ch Edling and L M Ödkvist

From the Departments of Otolaryngology Pathology I and Occupational Medicine University Hospital Linköping S eden

(Received October 31 1979)

Abstract Exposure to oil mist used in metal work sometimes gives symptoms from skin and airways This study was performed to evaluate histological and functional respiratory tract disorders Six male lathe workers aged 31–64 years exposed to oil mist for 4–29 years were examined and compared with matched controls The investigation included case history ENT examination nasal mucociliary function routine blood tests IgE, RAST X ray of sinus and lungs and biopsy of the nasal mucosa The mucociliary test showed no difference between the groups However all 6 exposed workers had pathological histology findings in the nasal mucosa including lack of cilia, basal cell hyperplasia goblet cell hyperplasia, squamous metaplasia and subepithelial hyalinization The biopsies from the controls were mainly normal The remainder of the in estigations revealed no pathology The study shows that exposure to oil mist—even below the permitted threshold limit—may cause airway symptoms and histological signs comparable to a premature ageing

A growing concern about various health hazards in industry has led to a situation where patients more frequently visit a physician because of different symptoms that they attribute to occupational exposure

In ENT practice we often see patients complaining of nasal and sinus problems which according to the case histories could be related to environmental pollution e g industrial solvents or oil mist

Oil or oil-in-water emulsions are used in various operations in metal working in order to reduce friction or help eliminate metal particles Mechanical dispersion of oil from rotating instruments produces oil mist with particle diameters varying from fractions of a μm up to 10 μm Particles smaller than 5 μm reach the alveoli

The constitutions of the oils vary greatly containing mixtures of hydrocarbons with various additives such as bactericides and antioxidation drugs Heavy metals such as cobolt chrome and nickel are often included in varying amounts (Ahling 1977)

No reports have been found in the literature concerning changes in nasal mucosa exposed to oil mist The following pilot study was performed to evaluate histological or functional disorders from the respiratory tract with special regard to the nasal mucosa.

MATERIAL AND METHODS

Six male industrial workers were examined They performed tasks on eleven automatic lathes in a metal-shop measuring 1 760 m³ The materials processed were mainly brass steel stainless steel and aluminium The cutting oils in use for the last few years were of a mineral type with less than 6% aromatic hydrocarbons and hardly any additives According to the producer these oils were comparable to other non-synthetic cutting oils regarding the health effects The working space was well ventilated and measurements made by the factory inspectorate showed low oil mist values around 1 3–2.5 mg/m³ to be compared with the Swedish threshold limit value of 5 mg/m³ As a control group we selected 7 men matched for age and smoking habits but without exposure to oil mist (Table I)

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For the exposed group the test battery also included spirometry measuring FEV_1, VC, FVC, X-ray of the sinus and pulm, culture from the nasopharynx, ESR, WBCC, eosinophils, IgE and RAST-analysis of dust, cladosporium, alternaria and housedust mite.

RESULTS

The exposed men had worked for 4–29 years (mean 15 years) as lathe operators. The histories disclosed nasal symptoms in 5 of 6 workers starting some years (1–14, mean 7) after their first occupational exposure to oil mist. Three of them had a marked relation to time of work, getting better during holidays. The two workers with the most pronounced symptoms had been treated earlier for recurrent sinuitis diagnosed by X-ray. Only one worker suffered from a cough with expectorate and conjunctivitis beside his nasal symptoms. There was no history of allergy except in one subject whose nasal symptoms were accentuated in early spring.

The clinical examination of the patients revealed grossly normal nasal mucosae. A minor polyp was found in one nose, and mucopurulent secretions were found in three others. One patient had a pronounced deviation of the septum, probably due to a trauma 20 years earlier; however, the history of nasal blockage was only 5 years. In the controls the histories and examinations revealed no pathology.

The mucocilary function did not differ markedly between the two groups according to the saccharine test. The flow rate is said to be about 0.46 cm per min, with very wide ranges. The clearance time can thus be expected to average 20 min. In both the exposed group and the control group there were 2 subjects with high values as a sign of reduced function (Table II).

All histological slides were examined on several occasions and without any knowledge of neither the clinical history or of previous exposure to oil mist. The results are summarized in Table III.

Table II

Patient number	Nasal symptoms	Bronchial symptoms	Interval between start of work and onset of symptoms (years)	Correlation to work	Mucociliary clearance (min)
Exposed group					
1	+	–	14	yes	17
	+	–	7	?	27
3	+	+	7	no	∞
4	+	–	1	yes	21
5	–	–	–	–	17
6	+	–	4	yes	50
Control group					
1	–	–	–	–	[illegible]
2	–	–	–	–	17
3	–	–	–	–	35
4*a*	–	–	–	–	∞
4*b*	–	–	–	–	∞
5*a*	–	–	–	–	6
5*b*	–	–	–	–	31

Control group

In all patients but one the nasal mucosae had major areas lined with a pseudostratified ciliated columnar epithelium (Fig 1) In one specimen (No 4*a*) squamous metaplasia had occurred and in another (No 5*b*) a rather low non-ciliated epithelium with cubique cells was present In one specimen (No 1) the nasal mucosa was lined with a non-keratinized stratified squamous epithelium

Table III

A = Pseudo-stratified ciliated epithelium present B = Rather low pseudo-stratified epithelium with no or few cilia or basal cell hyperplasia or goblet cell hyperplasia. C = Squamous metaplasia. D = Stratified non-keratinized squamous epithelium

	A	B	C	D
Exposed group				
1	×	×	×	
2		×	×	
3				×
4				×
5	×	×		
6		×		
Control group				
1				×
2	×			
3	×			
4*a*	×			
4*b*	×			
5*a*	×			
5*b*	×	×		

Exposed group

Histological changes in the nasal mucosae were observed in all patients although 2 still had areas with ciliated pseudostratified epithelium However these 2 patients also had goblet cell hyperplasia (No 5) and basal cell hyperplasia (No 1)

In 2 cases (Nos 3 & 4) the epithelium was replaced by a non-keratinized stratified squamous epithelium (Fig 2) A marked goblet cell hyperplasia (Fig 3) and squamous metaplasia were found in 2 patients (Nos 1 & 2) and in case No 6 a very low non-ciliated cubique epithelium was found A sub-epithelial hyalinization was also present in this mucosa (Fig 4) There was no sign of severe inflammation in any of the examined mucosae

One patient (No 4) refused both X-ray and spirometric examinations Spirometric results showed lower values than predicted in 3 of the 5 examined workers (including the one with a cough) (Table IV)

Pulmonary X-ray was normal in all cases X ray of the sinus revealed anthral clouding in those 2 patients with histories of sinusitis but was normal in the others

Blood samples were normal concerning ESR and WBCC The RAST analysis showed a slight increase in dust and mites in 2 different

Fig 1 Photomicrograph Nasal mucosa lined with pseudostratified, ciliated (arrow) columnar epithelium (toluidine blue 760)

patients one of them also having a somewhat increased number of eosinophils in his blood None of them had a positive history for dust allergy No culture with pathogenic bacteria was found

DISCUSSION

For 5 of the 6 patients we succeeded in finding matching persons to include in the control group for one the match is not entirely perfect due to a difference in smoking habits

In the working environment oil not only comes in contact with the respiratory airways but also with the skin Oil exposure can cause skin affections such as dermatitis or folliculitis

Table IV *Spirometric results (in % of predicted)*

Pat no	FEV	VC
1	103	101
	84	78
3	74	75
4		
5	77	97
6	130	99

Table II

Patient number	Nasal symptoms	Bronchial symptoms	Interval between start of work and onset of symptoms (years)	Correlation to work	Mucociliary clearance (min)
Exposed group					
1	+	−	14	yes	17
2	+	−	7	?	27
3	+	+	7	no	∞
4	+	−	1	yes	1
5	−	−	−	−	1?
6	+	−	4	yes	50
Control group					
1	−	−	−	−	18
2	−	−	−	−	17
3	−	−	−	−	35
4*a*	−	−	−	−	∞
4*b*	−	−	−	−	∞
5*a*	−	−	−	−	6
5*b*	−	−	−	−	31

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	A	B	C	D
Exposed group				
1	×	×	×	
2		×	×	
3				×
4				
5	×	×		
6		×		
Control group				
1				
2	×			
3	×			
4*a*	×		×	
4*b*	×			
5*a*	×			
5*b*	×	×		

Exposed group

Histological changes in the nasal mucosae were observed in all patients although 2 still had areas with ciliated pseudostratified epithelium However these 2 patients also had goblet cell hyperplasia (No 5) and basal cell hyperplasia (No 1)

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Table IV. *Spirometric results (in % of predicted)*

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1	103	101
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3	74	75
4		
5	77	97
6	120	99

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1	+	–	14	yes	17
2	+	–	7	?	27
3	+	+	7	no	∞
4	+	–	1	yes	21
5	–	–	–	–	12
6	+	–	4	yes	50
Control group					
1	–	–	–	–	18
2	–	–	–	–	17
3	–	–	–	–	35
4*a*	–	–	–	–	∞
4*b*	–	–	–	–	∞
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	A	B	C	D
Exposed group				
1	×	×	×	
2		×	×	
3				×
4				×
5	×	×		
6		×		
Control group				
1				×
	×			
3	×		×	
4*a*	×			
4*b*	×			
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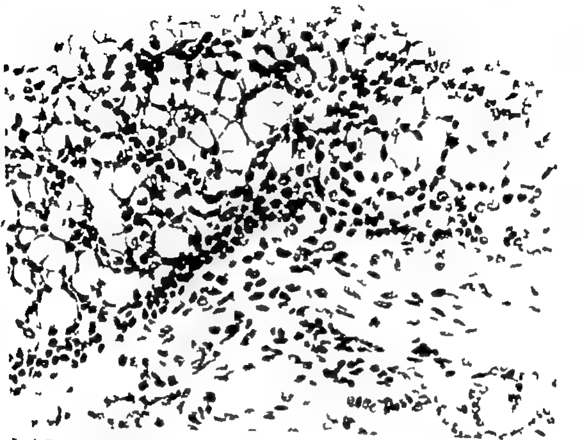

Fig. 3. Photomicrograph. Nasal epithelium with goblet cell hyperplasia but cilia are present as well (H & E, 390)

is much longer when the oil has been inhaled than if the oil merely passes through the nose.

The spirometry test in our 5 exposed patients did show lower values even when the patient did not have any respiratory symptoms. Weill et al. likewise reported that the spirometry performed in their cases showed values lower than anticipated from the case histories.

The saccharine test showed that the mucociliary clearance could not be proven worse in the patient group than in the control group. Presumably the saccharine test is too rough for the subtile mucosal changes.

Compared with those in the control group the morphological changes observed in the nasal mucosae of the exposed patients were rather prominent (Table III). The mucosae in the actual area (inferior turbinate) is supposed to be lined by a pseudostratified ciliated columnar epithelium (Bloom & Fawcett, 1970). This was the case in only 2 patients who also presented areas with a low, nearly nonciliated respiratory epithelium. It is reported that various agents, such as chromium, wood dust, copper salt dust and nickel, can induce morphological changes in the nasal mucosa, as well as functional disturbances (Gomes, 1972

Fig 2 Photomicrograph. Stratified squamous epithelium displaying hyperplasia but no atypia (H & E ×190).

(Johnsson & Wilson 1979) A high incidence of skin cancer especially scrotal cancer has been reported (Kipling 1974 Waldron 1975 1977) In a group of men with scrotal cancer exposed to oil an excess of subsequent primary tumours was found in the larynx bronchus lip stomach and skin (Waldron 1975) Two recently reported cohort studies have shown no increased mortality from cancer in total among men exposed to oil mist However both studies revealed an excess of cancer of the stomach and the colon (Decoufle 1978 Järvholm et al 1978)

The magnitude of the skin problems in workers exposed to oil mist fits well with the presence of nasal symptoms in the same category of workers as studied in our series Among our 6 patients 5 had a history of severe nasal symptoms Only one was free from nasal problems In the control group none had any nasal symptoms

Weill et al (1964) reported three cases of lipoid pneumonia in patients who had inhaled oil substances The etiology for these pneumonias is not necessarily closely related to nasal symptoms due to oil mist although the nasal mucosa and the bronchial mucosa are very similar Presumably the duration of exposure

symptoms do not appear until after 7 years work, supports that statement It must be taken into consideration however that oil mist is injurious for the workers and the pollution must be cut to a minimum.

ZUSAMMENFASSUNG

Metallarbeiter die mit Ölnebel in Berührung kommen, haben manchmal Haut- und Luftwegsbeschwerden. Die Verfasser haben die Luftwege einiger Metallarbeiter histologisch und funktionell untersucht. Sechs männliche Dreher im Alter von 31 bis 64 Jahren die im Zeitlauf von 4 bis 29 Jahren mit Ölnebel in Berührung kamen wurden untersucht und mit einer Kontrollgruppe verglichen. Die Untersuchung umfaßte Krankengeschichte, HNO-Untersuchung, Untersuchung der Nasenschleimhaute allgemeine Blutproben, IgE, RAST Röntgen von Nasennebenhöhlen und Lunge und Probeexzision der Nasenschleimhaut Der Mucociliärfunktionstest zeigte keinen Unterschied zwischen den beiden Testgruppen. Dagegen hatten die sechs Arbeiter die mit Ölnebel in Berührung gekommen waren pathologische und histologische Veränderungen der Nasenschleimhaut Wie Wegfall von Flimmerhärchen, Basalzellenhyperplasie Becherzellenhyperplasie Scheibenepithelmetaplasie und subepitheliale Hyalinisierung. Die Befunde bei der Kontrollgruppe waren meistens normal Die übrigen Untersuchungen wiesen keine Veränderungen auf Die Untersuchung zeigt, daß Personen die mit Ölnebel in Berührung kommen auch wenn die zugelassene Höchstgrenze nicht erreicht wird Symptome aufweisen, die auf ein vorzeitiges Altern der Schleimhäute hinweisen Außerdem können Luftwegsbeschwerden auftreten

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Lars Ödkvist
Department of Otolaryngology
University Hospital
S-581 85 Linköping
Sweden

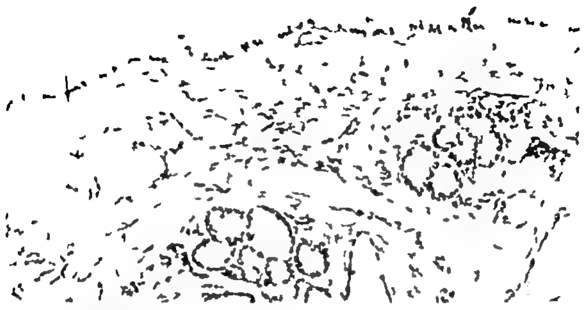

Fig 4 Photomicrograph A very thin nasal mucosa with subepithelial hyalinization (*arrow*) (H & E ×150)

Black et al 1974 Hadfield 1970 Askergren & Mellgren 1975 Torjussen & Solberg 1976)

The cilia of the mucosae of the exposed patients were partly or completely absent and the frequency of squamous metaplasia and hyperplasia was also higher in the exposed group Squamous metaplasia is thought by most authorities to be one possible pathway towards malignancy in respiratory tract mucosa (Robbins 1974) Metaplastic squamous epithelium and squamous epithelium were found in 4 of the exposed patients but also in 2 of the control group subjects None of these epithelia did show any atypia, as reported in patients exposed to nickel (Torjussen & Solberg 1976)

Impairement of the nasal mucociliary clearance was recorded in 2 patients (Nos 3 & 6) who also presented a squamous epithelium and a low respiratory epithelium with only a few cilia respectively However as squamous epithelium was found in patients with normal mucociliary function (patient No 4 and control No 1) no conclusions can be drawn regarding any connection between the mucociliary function and the presence of squamous epithelium in the region of the inferior turbinates Our clinical tests did not show such great differences between the two groups as could be expected from the morphological findings This is probably partly due to the circumstance that the tests are dependent on the respiratory tract as a whole and partly to the fact that the nasal mucosa differs in different regions (Krajina et al 1975) Consequently there is probably a need for multiple biopsies.

The metaplasia and the other histological changes suggest that the influence of oil mist in the nasal mucosa induces premature aging

The workers studied were exposed to oil mist in concentrations below the ruling Swedish threshold limit value of 3 mg/m^3 The findings presented here might indicate however that this needs to be further reduced if the aim of protecting workers from adverse health effects is to be fulfilled

Case histories biopsy findings and spirometry results show that oil mist has a noxious influence on the mucosa in the nose and the bronchi The saccharine mucociliary clearance test indicates that the changes in the mucosa are not too severe and the fact that the

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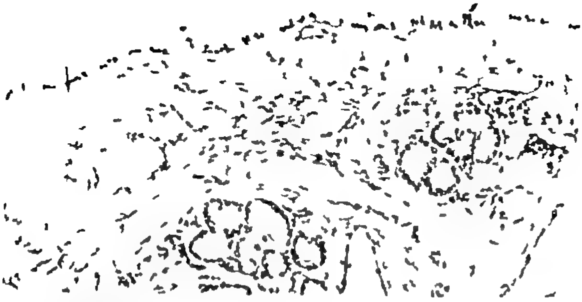

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Lars Ödkvist
Department f Otolaryngology
Univ rsity Hospital
S 581 85 Linköping
S eden

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A RELATIVE CONTRA INDICATION TO MEDIASTINOSCOPY

T Åberg

From the Department of Thoracic Surgery Uppsala University Hospital Uppsala S eden

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Abstract In a patient in whom a coarctation of the aorta had been successfully operated on 17 years earlier a life-threatening haemorrhage from a thin-walled, dilated collateral artery occurred in conjunction with a mediastinoscopy It is argued that diseases with increased mediastinal collateral vessels (coarctation of the aorta, cyanotic heart disease severe suppurative lung disease) are relative contraindications to mediastinoscopy even after a corrective procedure has been carried out.

Mediastinoscopy (Carlens 1959) is a well established and very valuable diagnostic procedure Its safety has been documented in several publications Asbaugh (1970) reviewed 9 543 cases and found a complication rate of 1 5% and a mortality of 0 09%

Very few local contra-indications to the procedure have been defined A case is presented in which a large arterial collateral vessel in the mediastinum was torn resulting in a life threatening haemorrhage The patient had previously been operated upon for a coarctation of the aorta. The patient a male was operated on at the age of 20 17 years before the present incident for a coarctation of the aorta An end-to-end anastomosis was made with a good result Several years after the operation he developed arterial hypertension He was re investigated and found to have a mean gradient across the anastomosis of less than 10 mmHg at rest and 40 mmHg during exercise at 900 kpm/min At the age of 37 he showed clinical and roentgenological signs of sarcoidosis To obtain tissue biopsies a mediastinoscopy was performed and a lymph gland was removed Postoperatively there was haematoma in the wound which increased and gave rise to a Stokes collar The mediastinum was re-explored Arterial bleeding was seen on the right side of the trachea about 3 cm above the carina. A tampon was inserted and the wound closed On X ray the mediastinum was now broadened On the suspicion that he was still bleeding he was sent to the Dept of Thoracic Surgery in Uppsala On arrival he had a well established Stokes collar mild stridor and increasing confusion An exploratory thoracotomy was made on the right side Several dilated collateral arteries were found in the muscles and chest wall In the mediastinum there was a large haematoma compressing the vena cava Copious bleeding was seen from the groove between the trachea and oesophagus The bleeding vessel was tortuous and looked like a dilated collateral This was ligated and the patient made an uneventful recovery Microscopy of the excised lymph gland revealed sarcoidosis

DISCUSSION

Several complications have been described following mediastinoscopy Injuries to surrounding structures (bleeding from pulmonary and innominate arteries from the superior vena cava and azygos vein perforations of trachea main bronchus and oesophagus in-

jury to the thoracic duct, vagus, phrenic and recurrent nerve injuries, pleural injuries (resulting in pneumothorax), infection and tumour seeding of the wound have been reported. These complications are very rare, however (Jepsen, 1971; Tucker, 1972) and may be minimized by knowledge of the limitations of mediastinoscopy. The present complication occurred because the nutrient vessel of the biopsied gland was thin-walled, tortuous and dilated. It is known that there are several conditions in which mediastinal and bronchial arteries may be dilated, namely coarctation of the aorta, any cyanotic heart disease and cases of severe, long-standing suppurative lung disease. Mediastinoscopy in such cases will probably run a higher risk of bleeding and should be avoided. The above-mentioned diseases are all correctable entities. The question then arises whether the collaterals persist even after the corrective operation. In the present case, it is evident that there were abundant collateral vessels in the chest wall and mediastinum in spite of the fact that the operation for aortic coarctation had been a successful one with only a minimal gradient remaining. One might speculate whether the need for collaterals had remained in this particular patient because of his hypertension and his minimal gradient, which during exercise increased to a haemodynamically significant level. Thus it seems that even in a successfully operated case of coarctation of the aorta, especially if the operation is undertaken late in life, collaterals may stay open long after the operation, thus increasing the risk of haemorrhagic complications at mediastinoscopy. Whether the same is true of cyanotic heart diseases and of suppurative lung disease is not known at present. However, it is common knowledge among heart surgeons that the bronchial arterial flow will diminish after a successful corrective operation in cyanotic heart disease, but that it will not always reach normal levels (This may be observed in re-operations of, for instance, cases of Tetralogy of Fallot.

From this single case history, no definite conclusions can be drawn. However, as mediastinoscopy is a diagnostic procedure which should carry negligible complications, it is advisable to regard coarctation of the aorta, cyanotic heart disease and severe suppurative lung disease as relative contraindications to the procedure, even if a corrective operation has been undertaken. If mediastinoscopy must be performed, it should be done in a hospital where an immediate thoracotomy can be safely carried out.

ZUSAMMENFASSUNG

Bei einem Patienten, an dem 17 Jahre früher eine geglückte Operation der Isthmus-Stenose der Aorta vorgenommen worden war, trat im Zusammenhang mit Mediastinoskopie eine lebensgefährliche Blutung auf. Meiner Ansicht nach ist bei Krankheiten mit erweiterten Kollateral-Arterien im Mediastinum (Isthmus-Stenose der Aorta, cyanotische Herzkrankheit, ernste eiternde Lungenkrankheit) Mediastinoskopie relativ kontraindiziert, auch spät nach einer operativen Korrektur.

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T. Åberg, M.D.
Dept. of Thoracic Surgery
Uppsala University Hospital
S-750 14 Uppsala
Sweden

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THE MUCOCILIARY ACTIVITY OF THE RESPIRATORY TRACT

I *Inhibitory Effects of Products of* Pseudomonas aeruginosa *on Rabbit Trachea in vitro*

Å Reimer [1] K Klementsson [1] J Ursing[1] and B Wretlind[2]

From the ENT Department and Department of Clinical Microbiology General Hospital Malmö and Department of Clinical Microbiology Karolinska Hospital Stockholm Sweden

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Abstract Bacteriological filtrates of three strains of *Pseudomonas aeruginosa* were compared with respect to inhibitory effect on ciliary movements and a quantitative difference was established between them The cilia inhibitory effect was strictly concentration-dependent and was resistant to heating. The ciliotoxic effect disappeared from filtrates after chloroform extraction The chloroform-extracted sediment was dissolved in physiological saline and the solution revealed an inhibitory effect on cilia. Involvement of endotoxin is not probable since *E. coli* endotoxin in a high concentration was not toxic. Partially purified *Pseudomonas aeruginosa* phenazine pigment as well as haemolysin inhibited ciliary activity and the effect was standardized in the present experimental system.

Inhibitors of ciliary activity released by microorganisms might contribute to colonization and penetration of the respiratory epithelium Organ cultures of ciliated epithelium from the respiratory tract of animals and humans have been used extensively in the study of respiratory viruses (Hoorn 1966 Hoorn & Tyrrell 1969) and mycoplasmas (Cherry & Taylor Robinson 1970 Collier & Baseman 1973 Gabridge et al 1974 Ping et al 1975)

Studies on the effect of bacterial toxins and living bacteria on ciliated cells are surprisingly few (Linton 1933 Hoorn & Löfkvist 1965 Denny 1974 Matsuyama 1974 and 1977)

Pseudomonas aeruginosa is of growing importance as a cause of pulmonary infections in hospitalized patients (Pierce & Sanford 1974) and is a common bacterium in the bronchi after tracheostomy (Espinoza et al 1974) and the middle ear with perforated drum (Palva et al 1971 Sadé & Halevy 1976). A variety of factors may contribute to its virulence and local effects (Scharmann 1976). The aim of this study was to investigate whether inhibition of ciliary activity could be recorded objectively and attributed to any particular toxins

MATERIAL AND METHODS

I *Bacteriological filtrates*

Pseudomonas aeruginosa strain PAKS-1 was isolated from a urine specimen at Karolinska Hospital in 1970 (Wretlind et al 1973*a*) PAKS-16 and PAKS-17 were protease-deficient mutants obtained after treatment of *Ps aeruginosa* strain PAKS-1 with a mutagen (ethyl methane sulphonate EMS) PAKS-16 produced less staphylolytic enzyme and lecithinase than the wild type strain PAKS-1 and PAKS-17 was defective in the formation of staphylolytic enzyme and lipase Both mutants produced exotoxin A (Wretlind et al 1977 Wretlind & Kronevi 1978)

Bacteriological filtrates were prepared from 12 hour broth cultures incubated in a shaker at 37°C and with a cell content of $1–2\times10^{9}$ per ml estimated by viable counts The medium contained the following proportions (w/v) of different substances peptone (Orthana) 1 0% beef-extract (Difco) 0 5% ; sodium chloride 0 3% glucose 0 1% and $Na_2HPO_4 \cdot 2H_2O$ 0 2% The cultures were centrifugated at

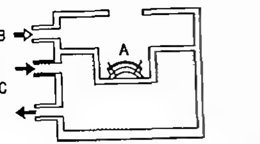

Fig 1 Experimental chamber with tracheal specimen placed in cup (A). Supply of conditioned air to keep specimen warm and moist during recordings (B). Temperature of test solution in cup controlled by circulating warm water during incubation (C)

2500–3000 r p m. and then filtered twice through a 0.22 μm Millipore filter The filtrates were tested for sterility and stored in 5 ml aliquots at −20°C

A fresh filtrate aliquot was used for each experiment Filtrates were serially diluted twofold with the medium Filtrates were heated in a waterbath at 56°C and 80°C for 30 min Medium was used in controls Chloroform extraction equal volumes of bacteriological filtrate and chloroform were mixed and shaken vigorously The mixture was allowed to settle out in a separating funnel The chloroform layer and the aqueous layer were separated and the chloroform phase was subjected to centrifugation at 2500–3000 r p m After evaporating off the chloroform any remaining sediment was dissolved in an equal volume of saline during heating (45°C) This solution and the chloroform-extracted bacteriological filtrate were then tested for cilia toxicity

II. *Haemolysin*

Pseudomonas aeruginosa haemolysin was purified from a mutant strain PAKS-51 This strain was obtained after treatment of *Ps aeruginosa* strain PAKS-1 with the mutagen (EMS) and selection for resistance to colistin. PAKS-51 was found to hyperproduce extracellular haemolysin phospholipase and deoxyribonuclease (Wretlind et al 1977) Strain PAKS-51 was cultivated in brain-heart infusion broth (Difco) supplemented with 50 mM glucose in indented shaking flasks at 37°C for 18 hours The haemolysin was purified from culture supernatant by boiling for 15 min ammonium sulphate precipitation and ethanol extraction according to Berk (1964) The purified product had an activity of 128 haemolytic units/ml against rabbit erythrocytes (Wretlind et al 1973*b*) The haemolysin was serially diluted twofold in physiologic saline

III *Pyocyanin (phenazine pigment)*

Pyocyanin was purified according to Armstrong et al. (1971) *Ps aeruginosa* strain PAKS-1 was cultivated in BHI broth with 50 mM glucose in indented shaker flasks The supernatant fluid containing pyocyanin was extracted with chloroform over night at +4°C The chloroform layer was separated from the mixture and evaporated The sediment was dissolved in chloroform and petroleum ether was added dropwise until blue needle-like crystals of pyocyanin appeared. Crystals were collected and dissolved in physiologic saline (1 mg/ml) This solution and serial dilutions were tested for inhibitory effect on ciliary activity

IV *Other tests*

A spectral comparison of the partially purified *Ps aeruginosa* haemolysin and phenazine pigment, as well as the chloroform extracts of PAKS-1 PAKS-16 and PAKS-17 was performed

Endotoxin (*E. coli* 026.B6 Difco lab Detroit, Mich USA) was dissolved in physiologic saline solution and tested at a concentration of 1 mg/ml

V *Mucociliary activity*

Tracheal preparations For each experiment a rabbit (weight 2–4 kg) without signs of infection was used The animal was killed with Nembutal® injected into an ear vein (approximately 100 mg/kg) The trachea was carefully prepared and a piece of about 5×8 mm was fixed with two needles to the convex surface

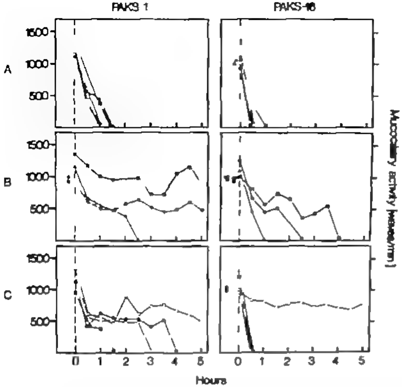

Fig 2 Effect of *Pseudomonas aeruginosa* strain PAKS-1 and PAKS-16 on mucociliary activity of rabbit trachea *PAKS 1* bacteriological filtrates stopped ciliary motility on five specimens within 90 min (A) Diluted filtrates (▲—▲ 1 2 ●—● 1 4 ■—■ 1 8) had a concentration-dependent effect (B) Heated filtrates (○—○ 56°C for 30 min ●—● 80°C for 30 min) had reduced effect. Chloroform-extracted filtrate (□—□) had almost no effect. Dissolved sediment from the chloroform phase (■—■) stopped ciliary motility within 90 min (C). *PAKS-16* bacteriological filtrates stopped ciliary motility within 60 min (A) Diluted filtrate (▲—▲ 1 2 ●—● 1 4 ■—■ 1 8) had a concentration-dependent effect (B) Heated filtrates (○—○ 56°C for 30 min ●—● 80°C for 30 min) stopped motility within 30 min The chloroform-extracted filtrate (□—□) had no effect but the dissolved sediment gained from the chloroform phase stopped ciliary motility within 30 min (■—■) (C)

of a silicon rubber tube the ciliated surface being inverted from concave into convex shape

Experimental chamber (Fig 1) The specimen when fixed to the rubber tube was placed in the bottom of a plexiglass cup The cup was warmed by a constant stream of heated water and the test solution was kept at 37°C during the experiments The milieu of the chamber was kept warm and humid by a constant supply of conditioned air The temperature was 36±1°C and the relative humidity >80% at the level of the specimen during recordings The chamber was adjustable perpendicularly by means of micrometer screws

Mucociliary activity The movements of cilia in the mucus layer were recorded by using an operating microscope a photodetector and an inkwriter (Reimer et al 1977) Recordings were made from the light-reflecting area on the convex surface of the mucous membrane Adjustment of the position of the specimen was sometimes necessary The recorded area was less than 0 25 mm² Recordings of 20 seconds duration were analysed manually and frequencies are given in waves/min

Test procedure A recording was made from a selected area with homogeneous ciliary activity as judged by ocular observation The cup was then filled with physiologic saline till the fluid surface was about 1 mm above the recorded area The liquid was aspirated and two more recordings were made immediately The cup was filled with test solution again in the same manner Recordings were performed every 30 to 60 minutes after aspiration of test solution and double rinsing of the specimen

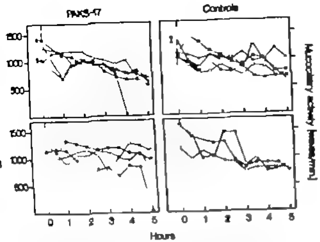

Fig. 3. Effect of *Pseudomonas aeruginosa* strain PAKS-17 and controls on mucociliary activity of rabbit trachea. *PAKS-17*: bacteriological filtrates resulted in slight reduction of activity and one specimen was ciliostatic after 4 hours (A). One filtrate heated for 30 min at 80°C (●—●) produced ciliostasis after 5 hours. The other heated filtrate (○—○ 56°C for 30 min) had no effect. Chloroform-extracted filtrate (□—□) had no effect. In contrast to PAKS-1 and PAKS-16 no visible substance was gained from the chloroform phase of PAKS-17 filtrate. Eventual sediment was dissolved in physiologic saline but the solution had no effect on ciliary movements (■—■) (B). *Controls*: tests with medium revealed slight reduction of mucociliary activity (A). The effect was about the same with heated medium (○—○ 56°C for 30 min, ●—● 80°C for 30 min) and with chloroform-extracted medium (□—□) (B).

with saline solution. The duration of each test was limited to 5 hours.

RESULTS

I. *Bacteriological filtrates*

The results of repeated experiments are illustrated graphically in Figs. 2 and 3.

PAKS-1. Filtrate of *Pseudomonas aeruginosa* strain PAKS-1 stopped motility after 50–90 min (Fig. 2 A). Diluted filtrate showed prolonged survival time of specimens and dilution 1:4 gave a constantly low frequency (Fig. 2B). Heated filtrates had less effect and the chloroform-extracted filtrate had almost none. After evaporating off the chloroform a visible sediment remained. Saline solution of the sediment revealed a weak blue colour and the solution caused ciliostasis after 90 min exposure (Fig. C).

PAKS 16. This filtrate stopped motility in most experiments within 30 min (Fig. 2 A). Dilutions showed a concentration-dependent effect (Fig. ?B). The toxic effect was not weakened by heating the filtrate but was eliminated by chloroform extraction. Just as for PAKS-1 filtrate a visible sediment remained after evaporating off the chloroform. Saline solution of the sediment revealed a weak blue colour and stopped ciliary motility within 30 min (Fig. 2 C).

PAKS 17. Tests with this filtrate resulted mostly in a slight reduction of activity and one specimen was ciliostatic after 240 min (Fig. 3). Control experiments were performed with chloroform-extracted and heated filtrate respectively. One filtrate heated to 80°C for 30 min produced ciliostasis after 300 min. Otherwise no toxic effects were seen. No visible substance was gained from the chloroform phase after chloroform extraction of bacteriological filtrate. Any remaining substance was dissolved in physiologic saline but the solution showed no toxicity on cilia.

Controls. Tests with medium revealed a slight reduction of activity (Fig. 3). Medium was heated and chloroform-extracted in the

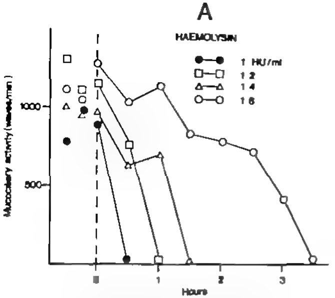

Fig 4 Effect of the partially purified *Pseudomonas aeruginosa* products haemolysin and phenazine pigment on mucociliary activity The haemolysin stopped ciliary motility within 30 min at concentration 1 haemolytic unit/ml (1 HU/ml) and dilutions showed a concentration-dependent effect (A) The phenazine pigment stopped ciliary motility within 30 min at concentration 1 mg/ml and the effect was less with dilutions (B)

same manner as filtrates and the tests revealed no toxicity

II–III *Partially purified P aeruginosa products*

Serial dilutions of the partially purified products of *Ps aeruginosa* showed a concentration-dependent inhibitory effect on cilia The haemolysin stopped ciliary motility within 30 min at a concentration of 1 HU/ml and dilutions showed gradually less effect At a concentration of 0 125 HU/ml ciliary motility stopped after about 3 hours (Fig 4 A) The phenazine pigment also showed an inhibitory effect on ciliary movements At a concentration of 1 mg/ml ciliary movements stopped within 30 min and dilutions had a concentration-dependent effect (Fig 4 B)

IV *Other tests*

A qualitative spectral comparison of partially purified *Ps aeruginosa* products and dissolved chloroform extracts from bacteriological filtrates showed that phenazine pigment PAKS-16 and PAKS 1 had corresponding peaks at 250 and 350 nm The haemolysin and PAKS 16 had a corresponding peak at 280 nm and a peak at this wavelength was possibly also seen for PAKS-1 and PAKS-17 (Fig 5)

The endotoxin of *E coli* had no inhibitory effect on ciliary movements Protease II (elastase) was purified according to Wretlind & Wadström (1977) No effect on mucociliary movement was found when 0 1 mg/ml was added to the tracheal specimen

DISCUSSION

In the preliminary assay bacteriological filtrates from a wild-type and two mutants of *Pseudomonas aeruginosa* were used The strains differed regarding production of extracellular products of various kinds Several investigations have shown that purified proteases from *Ps aeruginosa* elicited tissue damage after injection e g into skin or cornea (Kreger & Griffin 1974 Wretlind & Wadström 1977) Exotoxin A is cytopathogenic for animal cells and inhibits protein synthesis in such cells (Iglewski & Kabat 1975) A role for the phospholipases of *Ps aeruginosa* in pulmonary infections has been suggested (Liu

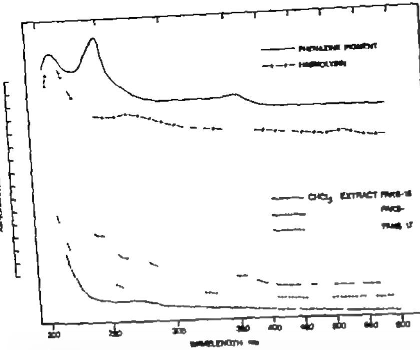

Fig 5 Absorption spectra for partially purified *Pseudomonas aeruginosa* haemolysin and phenazine pigment (above). Absorption spectra for the sediments gained from the chloroform phase after chloroform-extraction of bacteriological filtrates (below). All products were diluted with physiological saline till absorbance was in the measurable range. A comparison of products is qualitative only. Corresponding peaks are seen at 250 and 350 nm for phenazine pigment, PAKS-16 and PAKS-1. The peak at 270 nm for haemolysin is seen for PAKS-16 and possibly also for PAKS-1 and PAKS-17

1974) but conclusive evidence has not been presented.

When comparing filtrates from the three different strains a difference in ability to inhibit ciliary movements was evident. The difference in ciliotoxic effect could not be directly related to the known differences regarding production of extracellular products.

Since the effect of proteolytic enzymes is probably directed against cell junctions, the effect on ciliated mucosa might be a gradual release of ciliated cells. In spite of quantitatively few cilia, the mucociliary wave frequency was normal in investigations of human mucosal specimens (Reimer et al. 1978). The effect of *Ps. aeruginosa* filtrates was reproducible and strictly concentration dependent. Therefore some other mode of action was assumed. Further experiments showed that the cilostatic factors were resistant to heat (56°C and 80°C for 30 min) and the effect was recovered in the chloroform phase after chloroform extraction of filtrates.

The originally suspected extracellular toxins could be virtually excluded as contributors to the ciliostatic effect, since proteases as well as exotoxin A are inactivated by such treatments (Vasil et al. 1976).

A relation of occurrence of *Ps. aeruginosa* in respiratory infections and haemolytic effect on human erythrocytes was reported by Al-Dujaili & Harris (1975). It was also shown that the haemolytic activity was directly proportional to activity against alveolar macrophages (Al-Dujaili 1976). The present experiments with the partially purified *Ps. aeruginosa* haemolysin revealed a rapid effect on ciliary movements (Fig. 4 A).

Oxygen consumption of ciliated epithelium is high and has been shown to be related to

ciliary activity and intactness of epithelium (Gabridge 1975) Ciliary activity ceases rapidly in an anoxic environment (Reimer et al 1980) The phenazine pigment produced by *Ps aeruginosa* inhibits mitochondrial respiration (Armstrong et al 1971) A role of pigment was indicated by the observation of a weak blue tinge in the dissolved chloroform extracted sediments from filtrates of strains PAKS 1 and PAKS-16 but not of PAKS-17 The purified *Ps aeruginosa* phenazine pigment was capable of rapid inhibition of ciliary movement (Fig 4B)

The spectral comparison of the dissolved chloroform extracts shows several peaks for PAKS 1 and PAKS-16 Some of the peaks correspond to those of the purified *Ps aeruginosa* products haemolysin and phenazine pigment (Fig 5) Therefore a combined action of these products might be assumed A contributory effect of unknown substances is still possible

The relation of haemolysin production of *Ps aeruginosa* and occurrence in respiratory infections (Al Dujaili & Harris 1975) suggests that this toxin is of importance for virulence of *Ps aeruginosa* on ciliated epithelium and the present results support such an assumption The epithelial changes often seen in the mucous membranes in chronic otitis media (Hentzer 1972) might partly be explained by an effect of the *Ps aeruginosa* toxins that inhibit the activity of cilia.

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ZUSAMMENFASSUNG

Bakteriologische Filtrate von drei *Pseudomonas-aeruginosa* Stämmen wurden im Hinblick auf ihre inhibitorische Wirkung auf Zilienbewegungen verglichen wobei sich quantitative Unterschiede ergaben Die inhibitorische Wirkung war streng konzentrationsbedingt und widerstand dem Einfluß von Hitze Die zilientoxische Wirkung verschwand von den Filtraten nach Chloroformextraktion Das chloroformextrahierte Sediment wurde in physiologischer Kochsalzlösung gelöst und die Lösung erwies sich als zilieninhibitorisch Ein Einfluß von Endotoxin erscheint unwahrscheinlich, da *E. coli* in hoher Konzentration keine toxischen Effekte hervorrief Teilweise gereinigtes *Pseudomonas-aeruginosa* Phenazinpigment sowie auch Hämolysin hatten eine inhibierende Wirkung auf die Zilienaktivität und der Effekt ist in der hier dargestellten experimentellen Methodik standardisiert worden

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Å. Renner, M.D.
Dept. of Otolaryngology
University of Lund
Malmö General Hospital
S-21401 Malmö
Sweden

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palpation and other diagnostic tools are not necessary to reveal a complete palsy An in complete paresis of the trapezius muscle may be difficult to recognize however since the muscle is still contractile and visible hypotrophia may be lacking despite a considerable loss of strength Even electromyography may fail to confirm denervation since as much as 50% of the muscle strength may be lost without the occurrence of fibrillation (Knutsson 1979) Manual muscle testings do not activate separate muscles but merely groups of muscles *Nevertheless the strength of the lower* belly of the trapezius muscle can be tested fairly well as synergistic muscles are of less importance in the test manoeuvre of Daniels et al (1972) The superior belly may also be evaluated by manual muscle testing (Brunnström 1941) as has been verified electromyographically (Lundbladh & Moritz 1975)

Following radical neck dissection 23 of the patients (66%) in the present study exhibited a trapezius paresis at the postoperative examinations despite that the surgeon was unaware of any injury of the spinal accessory nerve Most of the patients were seen by the physio therapist the day after surgery Therefore it was not possible to establish whether the palsy had developed in connection to the surgical treatment or later secondarily to the inflammatory oedema of the wound At the final re-examination unusually performed several years after the neck dissection a major trapezius paresis persisted in 17% of the patients operated on In patients with major paresis the ability to abduct the arm and the abduction strength were equally reduced as in patients where the spinal accessory nerve had been sacrificed (Carenfelt & Eliasson 1980*b*) indicating a severe injury to the nerve

The importance of the trapezius muscle is not only to facilitate the abduction manoeuvre beyond the head The muscle also participates together with other muscles of the shoulder girdle in essential movements such as pulling pushing and throwing When the trapezius function is lost procedures such as eating dressing and driving a vehicle are not impossible to carry out but are frequently rendered far more difficult Pain in the shoulder region is not unusual and may appear also in patients with a minor trapezius paresis (Carenfelt & Eliasson 1980*b*) possibly due to the postural function of the trapezius muscle To minimize these physical sequelae the spinal accessory *nerve must be preserved at the neck dissec*tion whenever suspicious nodes do not approximate the course of the nerve However *the present results also indicate that the spinal* accessory nerve is most vulnerable and must be dissected with care

ZUSAMMENFASSUNG

Verletzungen des Nervus accessorius nach radikaler ‚neck dissection' treten trotz der Schonung des Nerven häufig auf Obwohl der Chirurg keine ernstlichen Verletzungen des Nervus accessorius bemerkte wurde eine Lähmung des Musculus trapezius bei 60 Prozent der Patienten während der Rekonvaleszenz beobachtet. Die Schulterfunktion besserte sich bei den meisten Patienten, aber eine stärkere Lähmung mit dem Verlust der wesentlichen Rotations- und Haltefunktion des Musculus trapezius bestand weiter bei 17 Prozent der operierten Patienten Achtzehn Monate nach dem chirurgischen Trauma wurde keine weitere Besserung beobachtet.

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C. Carenfelt M.D.
Dept. of Otolaryngology
Sabbatsbergs sjukhus
S-10401 Stockholm
Sweden

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Conferences and Meetings

1981 Jan 12–16, Mar 2–6, May 4–8, June 1–5 Oct 19–23 Nov 30 – Dec 4 Temporal Bone Surgical Dissection Courses will be given in the new Temporal Bone Surgical Dissection Laboratory in Ann Arbor Further information Malcolm D Graham, M.D., Department of Otorhinolaryngology Kresge Hearing Research Institute, University of Michigan Medical Center Ann Arbor MI 48109 USA.

1981 Febr 14–20 Third International Workshop—Neurological Surgery of the Ear and Skull Base to be held in Newport Beach, California, USA. Inquiries should be addressed to Derals E. Brackmann, M D., Neurological Workshop, c/o Ear Research Institute, 256 South Lake Street, Los Angeles, CA 90057 USA.

1981 Mar April, June, and Oct Temporal Bone Surgical Dissection courses in Los Angeles, California, USA. Further information Antonio De La Cruz, M.D Director Temporal Bone Surgical Dissection Course, Ear Research Institute, 256 South Lake Street, Los Angeles, CA 90057 USA.

1981 March 1–7 Departments of Otolaryngology of University of Toronto and University of Pittsburgh announce Winter Meeting 1981—Mont Trembland Lodge. Addr Dr William Crysdale, *Suite 6118, 555 University Avenue, Toronto, Canada* M5G 1X8

1981 March 22–27 Second International Conference on Cholesteatoma and Mastoid Surgery will be held in Tel Aviv Israel. Further details Professor J Sadé, 2nd International Conference on Cholesteatoma and Mastoid Surgery P O Box 16271 Tel-Aviv Israel.

1981 April 26 – May 2. Post Graduate Course in Ear Surgery to be held at the University of Nijmegen, The Netherlands. Further information from Prof Dr P van den Broek, University Hospital Sint Radboud, Department of Otolaryngology Philips van Leydenlaan 15 6500 HB Nijmegen, the Netherlands.

1981 April 28 – May 1 Conference on Health Education about Cancer to be held in Brisbane, Australia. Conference secretariat. Conference Secretary Australian Cancer Society inc., GPO Box 4708 Sydney N.S.W 2001 Australia.

1981 July 19–24 12th International Congress of Chemotherapy to be held in Florence, Italy Further information Secretariat, 12th International Congress of Chemotherapy Via della Scala, 10 50123 Florence, Italy